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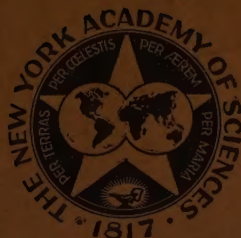
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SECOND CONFERENCE ON THE MECHANISMS
OF CELL DIVISION

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* This series of papers is the result of a conference entitled *Second Conference on the Mechanisms of Cell Division* held by The New York Academy of Sciences on March 3 and 4, 1960.

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THE SECOND CONFERENCE ON MECHANISMS OF CELL DIVISION

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The primary purpose of a conference as sponsored by The New York Academy of Sciences is to stabilize a plateau of knowledge in a selected field through the presentation of papers by investigators recognized for their ability and stature and to stimulate discussion among their peers. The resulting monograph, usually published in the *Annals* of the Academy, then consolidates the information presented and discussed at the conference.

It would be difficult indeed to find a more intriguing and exciting event in life than the reproduction of a cell: either plant or animal. With this thought in mind, the first conference on *The Mechanisms of Cell Division*¹ was organized and held in 1948 for the purpose of summarizing the areas that up to then, had been the most productive. Thus a foundation was established and the knowledge consolidated by those chiefly responsible for the development of their particular facet of the problem. The experimental approach to the study of cell division was well launched. Among these experimental approaches were: tissue culture, micrurgy, applications of high hydrostatic pressures, ultracentrifugal fields, and the production of centrifugal merogones. In addition, there were the applications of mitotic poisons, including the new radiomimetic chemicals, as well as chemical agents that uncouple oxidative phosphorylation in addition to the traditional metaphase blockers such as colchicine. The effects of radiation on cellular structures were being recognized, in particular radiation effects that might be induced internally through the action of radioisotopes.

Now, 12 years later, most of the information presented in this second monograph was either unknown or only starting in 1948. For example, electron microscopists were beginning to explore the fine structure of cells. Now, the avenues of fine structure analysis of cellular systems have been established and opened for handling incredible volumes of traffic. The use of tritiated thymidine in the study of chromosomal replication was unknown. Instrumentation for exploration of anisotropic structures by polarization optics as well as for inducing microbeam irradiation was perfected in recent years. Perhaps most important is the impact of the Watson-Crick structure of DNA and its proposed mechanism of replication. The past decade has been unusually productive, for example, even the spindle-aster complex of marine invertebrate eggs has been isolated.

Thus in this *Second Monograph on The Mechanisms of Cell Division* we see summarized such topics as the action of microbeam irradiation on chromosomal and spindle components; proteins and the anisotropic organized structure of the spindle; fine structures of chromosomes and spindle; autoradiographic studies on chromosomal replication; action of heavy water on cell division; nucleocytoplasmic relations; energetics of cell division; furrow induction; induction of synchrony in cell division; cell population kinetics; feed back

mechanisms; effects of hormones; macromolecular components and interactions of various types; and the regulation of mitotic rates, to name a few.

The study of cell division can be divided profitably into two categories: (1) Karyokinesis, and (2) cytokinesis. Strangely enough, perhaps more is known about the apparently more complex karyokinetic processes. Through applications of tritiated thymidine, the time for chromosomal replication is unquestionably narrowed down to the interphase. Since the first conference on cell division much has been said in recent years about DNA, in particular its structure, possible mechanisms of replication, the coding of information, and the synthesis of coded RNA. Not so clear are the mechanisms of gene action, although certain recent studies on the serial arrangement of amino acids in atypical hemoglobins are highly suggestive. There is in this area, however, the beginning of one of the most exciting developments in modern biology.

Through this *Second Monograph on The Mechanisms of Cell Division*, we have reached a second plateau. Plans must now be set for publication of a third monograph when advances in the field justify its appearance.

Future conferences on the mechanisms of cell division will probably stress the transmission of genetic information from cell generation to cell generation. What are the cyclic variations in a cell's potential to synthesize proteins, among other things, during mitosis? To what extent is mitosis a precise device for the partitioning of genetic material? What factors are responsible for mistakes in mitosis, which in some instances lead to aneuploidy? What effects do alternating exposures of chromosomes to cytoplasmic environments, which occur during mitosis, have on the masking or unmasking of gene action? Obviously, answers to these questions might brighten the horizon of cell differentiation.

What else can we expect in future developments? Perhaps, foremost is our present ignorance of what it is that trips a chain of events in a cell that culminates in karyokinesis and cytokinesis. To what extent does the centrosome control division? Can the action of a centrosome be modified by various experimental procedures? Can it be transplanted? One possibility stands out rather clearly, and this deals with recently devised procedures and instrumentation for the microsurgical transplantation of chromosomes, nucleoli, nucleolar-chromosomal complexes, and other subcellular structures from the nucleus of one cell into the nucleus of another cell. The microsurgical modification of chromosomes, such as the creation of translocations, will undoubtedly play an important part in future research.

The universal fourth dimension in biology, notably time, unfortunately has taken its toll. The following distinguished biologists who participated in the first conference and who contributed in the monograph are no longer with us: Edwin G. Conklin, Robert Chambers, E. Newton Harvey, Michael Levine, G. H. A. Clowes, Jonas S. Friedenwald, as well as Roy Waldo Miner, who edited the first monograph. Fortunately, through their students and publications, their imprint on biological thinking will remain with us and should serve as an inspiration to future generations of students engaged in unravelling still further the mechanisms of cell division.

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INTRODUCTORY REMARKS

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During approximately the first forty years of this century biologists whose concerns were with the dynamic and molecular events underlying cell life called themselves general physiologists. Following the direction established by Claude Bernard, they sought to examine by chemical and physical methods those phenomena that seemed to transcend phylogeny, that is, to be common to all plants and animals. A central problem for general physiologists was cell division. However, the general physiologists have become sadly scattered in our time . . . the term hardly retains its original significance, for whole areas of their originally unique interest have been occupied by specialists in particular types of experimental investigation. Thus most biochemists, biophysicists, and many cytologists working at the ultrastructural level, are today studying aspects of general physiology. It is interesting that general physiology often, albeit not always, meant "cellular physiology."

There is, however, no need to mourn the passing of the name. The great growth of biochemistry and biophysics was quite inevitable and wholly appropriate. One looks forward to an eventual reunion of the disciplines that emerged separately from general physiology, perhaps under some such name as "molecular biology," under conditions that will permit the recovery of the older "general" concepts from a higher level of physicochemical sophistication.

Certainly, however, many of the special interests of general physiology did cease being objects of active investigation in the course of its fragmentation. A glance at one of the distinguished textbooks of the 1930s will provide many examples. Cell division, however, has emphatically *not* suffered such a fate. The topic has remained a central concern of the best biological minds almost continuously since the beginnings of experimental cell research.

The explosive growth of biochemistry and biophysics has not withdrawn interest from this perennial general physiological problem; rather, each discipline has lent, when they became available, important new insights and techniques to its investigators.

It is of interest to inquire why cell division should have remained a major independent concern of biology for so long a time and, to inquire further, why its study did not become the province of a guild of specialists as did so many other problems in cellular biology. The conceptual form of the problem and its mystery have, instead, remained distinct from any single technical formulation, and contributors continue to approach it from as many points of view as there are techniques in experimental biology.

I shall put your minds at ease by saying at once that I have no intention of making such an inquiry here. With your indulgence, however, I shall give what I believe to be two of the most important factors; it is these, furthermore, which, in addition to the technical advances of the past decade, have motivated the publication of this monograph.

Despite the multitudinous variations in detail discovered by cytologists, despite the obvious differences between its course in plants and that in animals,

and despite the discoveries of seemingly "autonomous" behavior on the part of one or another player in the chromosomal charade, the mitotic cycle retains a universality in its major features matched only by such properties of life as the presence of nucleic acids and porphyrins, of potentials across cell membranes, and by the cell theory itself. There exist many explanations for this universality. The plan of cell reproduction is in itself so supremely "logical," as Daniel Mazia points out, that one tends to forget that such reproduction need not necessarily occur by mitotic division as we know it. Of all the explanations offered, one seems especially attractive to contemporary students of mitosis; that is, that mitotic cell division must have appeared very early in the evolution of cellular life on this planet, and that its fundamental pattern has remained impressed (perhaps because of a unique economy of operation) on all descendants of those protocells. Such a proposal has the merit, as N. G. Anderson stresses, that mitosis must be accomplished with materials and by molecular processes that, like mitosis itself, transcend phylogeny.

Models for the assembly and operation of the mitotic machine thus continue to be made, just as they were during the flowering of general physiology; the difference is that several decades of contact with those lusty infants, biochemistry and biophysics, have sharpened the simplifying assumptions, and improved greatly the physical sophistication of the model-makers. One of the primary purposes of this monograph has been to provide an opportunity for the model-makers to discuss their products and the significant quantities of supporting data that have accumulated within the last decade.

The unique continuity of cell division research has had another, and perhaps more obvious, basis. Discovery of the mechanisms of cell division is a matter of the utmost practical importance. Ours is an age when research in pure science has largely lost its innocence. Whether for good or for evil, disinterested investigation of nature's most obscure and complex molecular phenomena has become a matter of public concern and support. A small but significant example of this is the generous support given to the publication of this monograph by both industry and private charity. Two matters of the greatest public concern are problems whose final solution will depend upon much deeper insight than is now available into the mechanisms of mitosis: I refer, of course, to neoplastic disease and to radiation injury. So various, however, are the experimental attacks upon neoplasia and upon radiation damage and resistance thereto, that almost the only conceptual structure to which the mountains of data can conveniently be referred is that concerning normal cell division and its control. The central position of this structure, as it were, amidst an outpouring of work by physiologists, pathologists, biochemists, biophysicists, cytologists, and geneticists has prevented the fragmentation and guild formation that overtook other problems first formulated by the general physiologists.

Clearly, a body of fact and theory so important to the development of useful results in applied biology must be dusted off and displayed at intervals of about 10 years, if that body is to enjoy the continual reshaping required. This, then, is a second motivation for the publication of the monograph.

The preceding remarks have been concerned with the importance of theories of cell division in modern biology. To those remarks I must now add that,

while the problem appears by no means to be close to solution, significant progress has been made during the past decade in experimental technique.

Particularly striking have been advances in microscopy, that indispensable method in cell research. We have devoted a not inconsiderable portion of this publication to papers that represent those advances. Phase-contrast and polarization microscopy and, of course, microcinematography have enjoyed fundamental improvements in theory and application, and our understanding of the mitotic machinery has been enriched thereby. The works of such investigators as S. Inoué, M. M. Swann and J. M. Mitchison, G. Östergren, A. Bajer, and the members of the biophysics group in Chicago, Ill. have stimulated a quantity of new, more disciplined speculation about the operations of the mitotic apparatus.

No mention of advances in microscopy would be complete, however, without appropriate prominence for the electron microscope. It seems strange to reflect, when we are in daily contact with such distinguished specialized journals as *The Journal of Biophysical and Biochemical Cytology* and the *Journal of Ultrastructure Research*, that contemporary techniques in high-resolution electron microscopy are no more than 10 years old. While direct examination of mitotic structures (other than chromosomes, concerning which there is already a considerable literature) has only recently begun, it is a truism that no integrated activity of cells can adequately be described now without reference to those submicroscopic structures that have been revealed by the electron microscope as constant components of the cytoplasm.

The development of cytochemical technology has added its share to progress in the study of mitosis. The acceptance, if perhaps with reservations, of mass isolation methods has encouraged widespread analysis of cellular phenomena in terms of the biochemical properties of isolated cell fractions. The outstanding example of direct application of such methods to mitosis has been, of course, the brilliant accomplishment of Mazia and of K. Dan in isolating the intact mitotic apparatus from populations of dividing cells, and the further exploitation of their specially-contrived techniques by able students and collaborators.

Submicromanipulative and manometric methods have been developed extensively in the past 10 years, so that growth and the synthetic activities of single cells can now be followed through complete division cycles. These data have contributed to a sharpening of the analysis of the division cycle as a whole and to the statement (and perhaps abandonment) of the "trigger" hypothesis. Very valuable independent evidence in these matters has been obtained from the remarkable studies on populations of synchronously dividing microorganisms. These have also served usefully in initiating formal analyses of cell population kinetics.

The immense efforts that have been devoted to studies on stimulation and inhibition of cell division in such diverse systems as tissue culture, wound-healing, limb- and organ-regeneration, tumors, and invertebrate eggs have not been without direct benefit in the analysis of the more general features of

mitosis. Especially helpful have been biochemical insights into metabolic transformations accompanying the capture and storage of free energy by cells, and analysis of the roles of energy-yielding processes in various steps of the mitotic cycle. Highly stimulating surveys of the entire body of data relating to the control of cell division have been published by M. M. Swann. A number of contributions representing this area appear in this publication.

Finally, the gratifying increase of knowledge about the structure and behavior of macromolecules, especially of proteins and nucleic acids, has had direct and important effects upon the study of cell division, for several important lines of current investigation are aimed toward the identification of specific macromolecular and particulate fractions involved in the assembly and operation of the mitotic apparatus. Furthermore, advances in the study of polyelectrolytes and their interactions have been forced without pause into the service of investigators seeking mechanisms for those assemblies and operations. Thus many of the properties of classical mitotic poisons are being reinterpreted, by such investigators as Anderson, in terms of polyelectrolyte theory.

This brief sketch of progress in the study of cell division is necessarily incomplete. Our belief is that at least a fair proportion of the main lines of advance are represented in the discussions that follow, and that in the course of their presentation, the current shape of the cell division problem will emerge. It is clear from the titles alone that experimental attack upon the problem has never before been so vigorous nor so competently staffed. Our contributors, who must here represent a far greater number of investigators situated in every corner of the civilized world who are making important contributions, represent in addition a multifaceted interest in a single phenomenon perhaps unprecedented in biological research. We cannot but echo the spirit of optimism that emerges from so many of the papers; it is our hope that this spirit will help to motivate even greater and more fruitful effort, so that by the time the third monograph on cell division is published by the Academy, substantial portions of our common problem will have been solved.

"... Can then physics and chemistry out of themselves explain that a pin's-head ball of cells in the course of so many weeks becomes a child? They more than hint that they can."

Reference

1. SHERRINGTON, C. 1953. *Man on His Nature*. 2nd ed. Doubleday. New York, N. Y.

CHANGES IN FINE STRUCTURE OF PARTS OF CHROMOSOMES AFTER LOCALIZED ULTRAVIOLET IRRADIATION

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A segment of a chromosome after being irradiated with the Uretz ultraviolet microbeam^{1,2} undergoes a prompt change in refractive index and appears optically negative instead of black with dark contrast phase microscopy.³ For convenience we have often called this phenomenon "paling," but this is a poor term since the changed area would undergo "darkening" when examined with a bright contrast phase microscope.

When the cells are fixed for cytological study, the locally irradiated areas do not stain with most nuclear stains or with methyl green in the pyronin-methyl green mixture, and react very faintly or not at all with the Feulgen method.⁴ Further study has shown that if sufficient time is allowed to elapse between irradiation and fixation (45 or 60 min.) the irradiated areas become completely Feulgen negative. Ultraviolet absorption studies show that the irradiated areas have a greatly diminished absorption at 2600 Å.⁵

The failure of the irradiated area to stain with the Feulgen and pyronin-methyl green methods and the decreased absorption at 2600 Å suggest that the nucleic acid had been greatly changed or lost from this portion of the chromosome. From the change in refractive index in the area one might suspect that it had lost some material. This inference is bolstered with preliminary interference microscopy measurements of optical path differences between irradiated and nonirradiated chromosomal regions that suggest a relative dry mass loss of about 15 per cent from the former (R. H. Haynes and M. Stodol-sky, personal communication).

For the last two years I have been studying the structure of the changed areas in the chromosomes with the electron microscope. To this end a convenient method was devised by which the partially irradiated cell could be found and sectioned for electron microscopy.⁶

The somatic cells studied were mesothelial cells growing in an epithelial sheet from explants of urodele heart in chick plasma diluted 1 to 9 in amphibian Tyrode solution.

Some of the findings with the electron microscope may be summarized as follows. In cells fixed with 1 or 2 per cent osmic acid solutions at several hydrogen ion concentrations I have not been able to find structural differences between the chromosomes in the irradiated and nonirradiated areas. In all of them the chromosomes appear as masses of short rods or of tiny black dots that sometimes form short chains; the spaces between the rods and dots are very pale and do not suggest any structure. The outlines of the chromosomes are exceedingly vague, and it is difficult to tell where the chromosomes end.

After several months spent with these negative experiments, I began to study the living cells with dark contrast phase microscopy during fixation. The first significant finding was that the pale, irradiated area, clearly visible as a bright zone with phase microscopy, disappeared as soon as osmic vapor

came in contact with the chromosomes; that is, the discontinuity in the dark chromosomes at the irradiated zones disappeared immediately so that the pale area could no longer be detected in the living cell.

In contrast to this was the result obtained with iodine vapor that turned the nonirradiated parts of the chromosomes brown but the pale, irradiated area did not change. Similarly, the irradiated area remained unchanged with formalin vapor. As a result, I have used a variety of formalin-containing and other fixatives on the partially irradiated cells. I shall mention only some of the findings with simple formalin solutions; the results obtained with form-

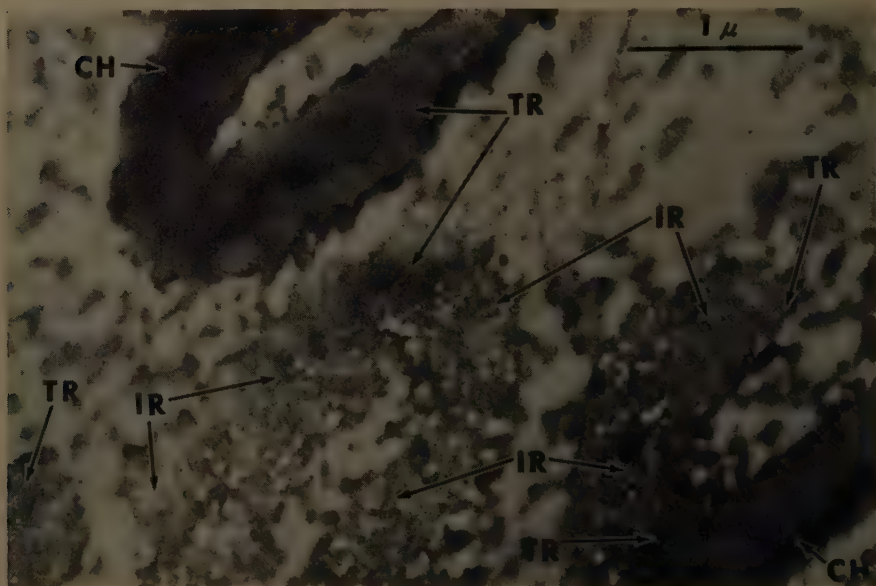


FIGURE 1. Electron micrograph showing effect of ultraviolet microbeam irradiation on parts of chromosomes of newt mesothelial cell: CH, unirradiated parts of two chromosomes; IR, irradiated portions of chromosomes showing large irregular holes in contrast to the homogeneous structure at CH; TR, transition zone with tiny holes between irradiated and non-irradiated portions. Fixation in 10 per cent neutral formalin in amphibian Tyrode solution.

aldehyde solutions containing heavy metals and some with various concentrations of osmic acid will not be considered here.

Most of the cultures were fixed for 30 min. in a mixture of 1 part of formalin (neutralized with $MgCO_3$) and 9 parts of amphibian Tyrode solution.

After this fixation the chromosomes have an entirely different appearance from that seen after treatment with osmic acid. Their outlines are distinct although there are occasional projections of small tubelike structures into the chromosomal substance. The body of the chromosome appears homogeneous in contrast to the vaguely outlined, dotted structure after osmic fixation and, in some cases, there is more or less crumpling due to compression in the cutting. Again—in contrast to the osmic acid fixed preparations that did not show any differences between irradiated and nonirradiated chromosomes—after

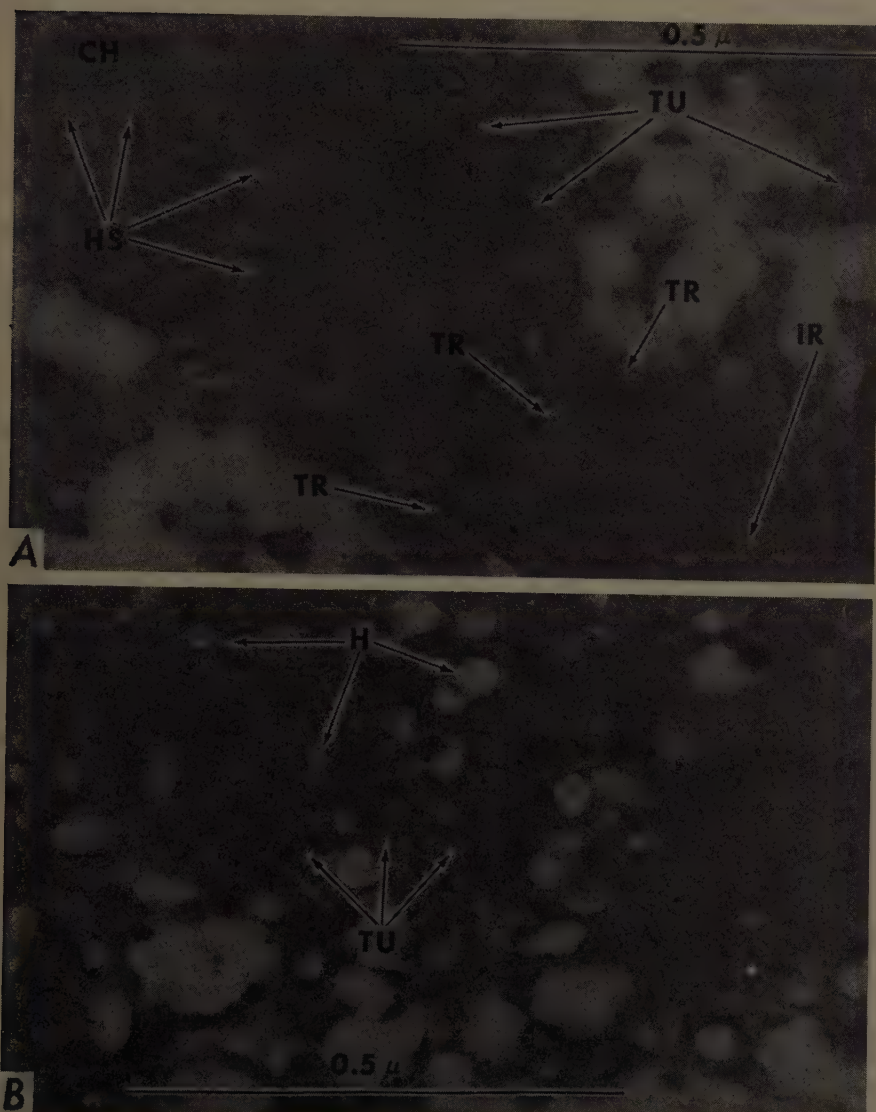


FIGURE 2. Electron micrographs showing effect of ultraviolet microbeam irradiation of part of a chromosome of mesothelial cell of amblystoma in tissue culture. (A) The unirradiated part of the chromosome (CH) contains homogeneous substance (HS) and tubules (TU); TR marks approximately the beginning of the transition zone between the nonirradiated and the irradiated portion (IR). There is decidedly less homogeneous material in the lower right hand corner of the chromosome than in the upper left. (B) This portion of the same chromosome is from the intensely irradiated part. It consists mainly of tubules (TU) and holes of varying sizes (H). The homogeneous substance shown in FIGURE 2A is apparently missing.

Fixation in 10 per cent neutral formalin in amphibian Tyrode. Stained *in toto* with 0.5 per cent phosphotungstic acid in absolute alcohol.

the formalin Tyrode solution fixation, the ultraviolet-irradiated areas are strikingly different. In them, the outlines of the chromosomes are no longer distinct, and a large number of holes of varying sizes are present. In the zone of junction between the irradiated and nonirradiated areas the holes are much smaller and of relatively uniform size (FIGURE 1).

Especially informative pictures are seen in formalin-fixed preparations when the cultures are passed through 0.5 per cent phosphotungstic acid in the dehydrating absolute alcohol before embedding. In such material the chromosomes have at least two main constituents; one of these consists of a finely granular substance; the other of irregular tube- or rodlike structures (75 to 300 Å in diameter, FIGURE 2). They are sometimes slightly curved or occasionally branching and some of them continue into the interchromosomal areas. They are apparently separated from one another by the finely granular paler substance. This material begins to diminish progressively in the narrow transition zone between the nonirradiated and the irradiated parts of the chromosomes and may be absent in the intensely irradiated zone (FIGURE 2, *B*).

It may be repeated here that the ultraviolet-irradiated parts of chromosomes do not stain with the Feulgen method or with methyl green and show greatly decreased absorption at 2600 Å. As the tubular or rodlike structures persist in the irradiated part of the chromosome, as seen in electron micrographs, it seems probable that DNA is one of the constituents of the homogeneous pale-staining material that disappears from the irradiated areas.

Differences in chromosome structure after localized ultraviolet irradiation are visible with the electron microscope in the formalin-fixed preparations. No differences have been found in osmic acid fixed cells.

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ASTER-ASSOCIATED PARTICLES IN THE CLEAVAGE OF MARINE INVERTEBRATE EGGS*

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Introduction

Recent advances in technique have made us aware of a number of individualized compartments in the cell cytoplasm that were not in the catalogue of the classical cytologist. The electron microscope has shown the basophilic, striated parts of cells to be highly varied and pleomorphic in structure—for example, endoplasmic reticulum (Palade and Porter, 1954) annulate lamellae (Rebhun, 1956), and chromatoid bodies (Ruthmann, 1958)—and has revealed such particles as microbodies in liver (Rouiller and Bernhard, 1956) and multivesicular bodies in rat ova (Sotelo and Porter, 1959). Refined methods of differential centrifugation have established the existence of lysosomes (de Duve, 1959). Tissue culture has revealed a small particle (Rose, 1957) whose behavior suggests a possible relation to lysosomes.

Concomitantly with this increased armamentarium of particulates in the cytoplasm have come indications of close morphologic associations (if not actual connections) between certain of these particulates, at least at certain stages in the life history of the cell. There is a likelihood that the Golgi apparatus and the endoplasmic reticulum are connected at certain times in the secretory process in pancreatic cells and probably in other cells as well, and the former may also be in constant interchange with the cell membrane. The endoplasmic reticulum appears morphologically connected to the nuclear envelope (Watson, 1955), which may be derived from it—and vice versa, depending on the portion of the cell cycle that one considers—and perhaps to the cell membrane. Certainly, different types of the ergastoplasm, such as endoplasmic reticulum and annulate lamellae in oöcytes are morphologically connected (Rebhun, 1959a). Again, the microkinetospheres in HeLa cells appear to fuse with pinocytosis vacuoles to form compound bodies (Rose, 1957), a process that may be very widespread (for example, in “droplets” in kidney; Straus, 1957).

I shall present in this paper some recent work that my associates and I and several other groups have been doing on certain particles that appear in many eggs and that exemplify certain points made above; namely, that they may at certain periods in the mitotic cycle be interrelated and may be derived from other better known cell organelles. These are the metachromatic α and β particles in eggs (see Rebhun 1959b for more complete bibliography). These particles can be stained in living cells with many basic dyes, and their history can be traced subsequently during mitosis and early embryogenesis. In a sense, these particles may be considered “new” or, more accurately, newly rediscovered. We have found work in Japanese by Iida (1942) and by Fischel (1899), both of whom reported granules stainable with neutral red in sea urchins which show some of the properties described below.

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Methods and Observations

Vital staining. A description of the two different techniques used in staining the particles in this study is important since observations with one technique differ in certain ways from those with the other. In the first technique (method I), unfertilized eggs of the surf clam, *Spisula solidissima*, were stained in increasing concentrations of methylene blue in sea water, not exceeding 1 part dye to 125,000 parts sea water. Eggs so stained show no apparent injury and show better than 99 per cent synchronized cleavage after fertilization, to approximately third cleavage, even if they have been standing unfertilized overnight (at 18° C.). The cytoplasm contains a number of dark blue particles which, if staining has not been prolonged past 4 hours, are from one fourth to 1 μ in diameter (the lower limit clearly being only a very crude estimate). They undergo a characteristic type of movement, which consists of short periods of rapid translation (during which time the particles may cover distances of 10 to 20 μ at rates up to 5 μ /sec.) interspersed with longer periods of relative quiescence (except for a slight Brownian movement). Upon fertilization, the particles begin to show a migration into the forming egg asters which, however, in early stages, is of variable extent in eggs of the same batch. As first cleavage approaches, more and more particles enter the astral regions and, by the time the first cleavage spindle is formed, very few particles can be found elsewhere (FIGURES 1 to 4) in any egg (Rebhun, 1959b). The motion of the particles into the asters is characteristic and consists of the same type of movement as seen in unfertilized eggs, but now is directed toward the centers. Specifically, a given particle may not move for minutes and may then suddenly show a spurt of movement, the direction of which is radial to the centers. This sudden motion, although radial, may occasionally be away from the center rather than toward it, although clearly the total distance of movement toward the center must predominate over that away from it. It is also important to note that this movement usually involves only single particles and that yolk and mitochondria or other stained particles that may appear sufficiently close to touch the one particle that will move do not become involved in the movement. After first cleavage, most of the particles gather on the peripheral pole of the blastomere nucleus (FIGURE 5), and the group of particles then divides into two groups (with different numbers of particles), which then outline the asters of the second cleavage spindle (FIGURE 6). This process is continued at least until fifth cleavage, past which we have not followed it in detail (Rebhun, 1959b).

The second method of staining that we have referred to above (method II) consists of staining the eggs in solutions of 1 part toluidine blue (or neutral red or Azure B) in 75,000 to 100,000 parts of sea water for from 0.1 to 5 min. Eggs are then washed by gentle centrifugation in a hand centrifuge until no more dye appears in the sea water (1 to 2 min. is sufficient). This procedure, the one used by the Belgian group of Dalcq, Pasteels, and Mulnard (see for example, Pasteels and Mulnard, 1957), has been used with normal eggs at all stages from before fertilization to after third cleavage and on eggs stratified by centrifugation at the same stages (*see below*).

If one observes eggs, stained as above, at any stage in cleavage immediately after staining, most of the eggs, at low power, appear relatively darkly and

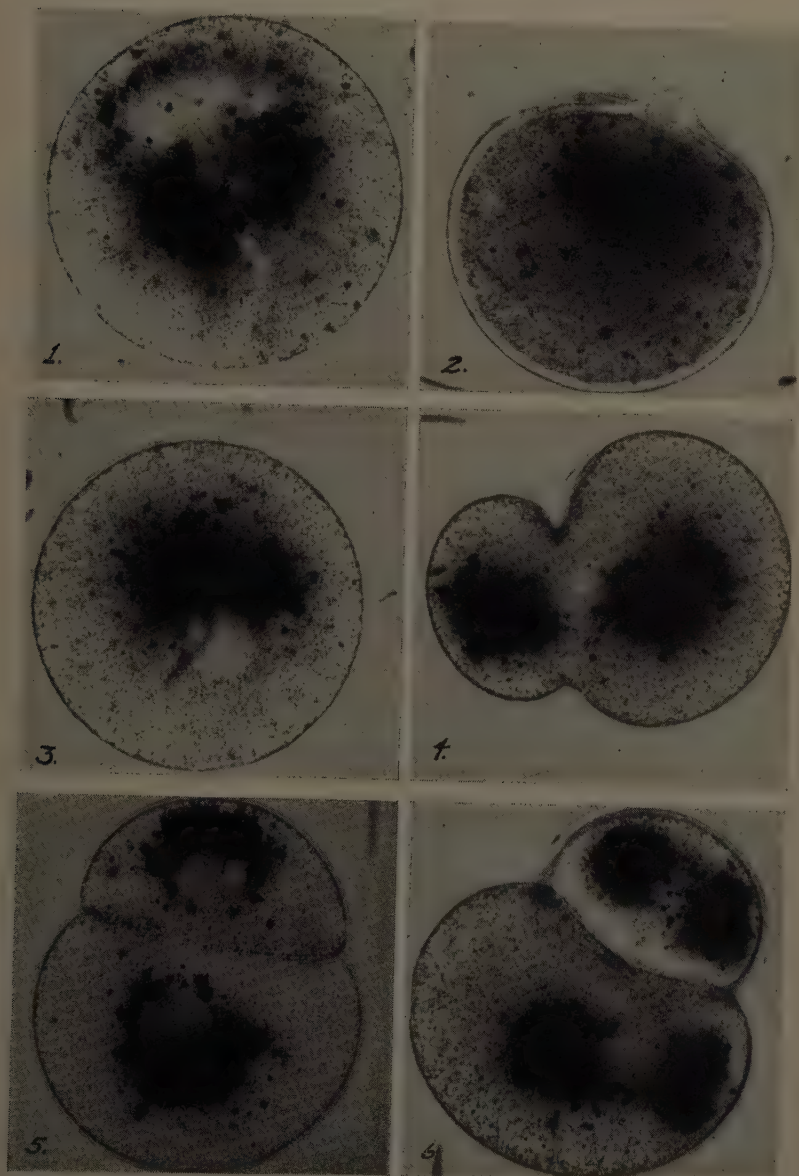


FIGURE 1. First polar body spindle forming in cell 15 min. after fertilization.
 FIGURE 2. Second polar body just being formed in fertilized egg at 40 min.
 FIGURE 3. Conjugation of pronuclei at 50 min. past fertilization.
 FIGURE 4. First cleavage almost completed at 60 min.
 FIGURE 5. Interphase between first and second cleavage at about 70 min.
 FIGURE 6. Second cleavage at about 80 min.

Eggs in FIGURES 1 to 6, stained by method I (methylene blue).



homogeneously stained. Under an oil immersion lens it may be seen that the cytoplasm contains many uniformly distributed small (less than one-fourth micron, that is, just at the limit of visibility) heavily stained particles (α -particles). In addition, however, in eggs that are stained in periods during which asters are present, larger, (one-half micron or more) less densely stained particles can be seen in the astral regions, although this is difficult to verify in every case because of the poor optical conditions in the particulate-strewn cytoplasm of the egg. In centrifugally stratified eggs, however, the occurrence of astrally located particles (β -particles) at this time can be verified. *Spisula* eggs may be stratified by applying forces of 8000 to 10,000 g for 3 to 4 min. and, if stained eggs are so treated, the metachromatic particles appear in two regions: one is the distal centrifugal region among the yolk particles and the other is in a layer just below the centripetally located lipid cap. Some of the particles, in addition to being spread in a layer, may be associated with the asters if these are present. In all cases, however, the particles, if watched periodically for the next 10 min. or so, may be seen to migrate into the asters which, if not still present after centrifugation, soon reform (FIGURE 7). Thus, since the particles are sublipid in distribution after centrifugation and show a migration into the asters, they satisfy the conditions used by Pasteels and Mulnard (1957) in *Barnea* (a lamellibranch related to *Spisula*) to define the particles as β particles. The centrifugal particles are the α particles.

In addition to the above experiments, eggs were first stratified at different stages in cleavage and then stained by method II. In all cases very similar distributions of particles were seen in these eggs compared to those stained before stratification; that is, a group of α particles appears in the centrifugal yolk region and a group of β particles appears in the sublipid layer as before.

If one follows eggs stained by method II at any time prior to pronuclear fusion, one notes that at about the time of formation of the first cleavage spindle, most of the uniformly distributed metachromatic material in the egg "disappears" except that in the astral regions that are now densely outlined with metachromatic particles (see FIGURES 8 to 11 for spindles in various stages of formation in stratified eggs that subsequently cleaved). It should be pointed out that if eggs are stained at first cleavage, they appear, as previously noted, relatively homogeneously stained with small particles and that the astral localization of β particles may be seen only with some difficulty in most eggs (in some eggs, no difficulty is encountered). In this case the egg "clears up" at about the time the second cleavage spindle forms, when the astral localization of all metachromatic material becomes clear.

FIGURE 7. Reassociation of β particles about aster in centrifuged egg. L = lipid cap, C = cortical granules, Y = yolk.

FIGURE 8. Centrosome (Ce) surrounded by β particles in egg in which nuclei have begun conjugation.

FIGURE 9. A stage similar to that in FIGURE 8, but a few minutes later.

FIGURE 10. Asters of a first cleavage spindle surrounded by metachromatic particles.

FIGURE 11. Cleavage furrow beginning. Note the upper hyaline layer (UH) beneath the lipid cap.

FIGURE 12. β particles gathering about asters in an unfertilized egg, activated by ageing. The germinal vesicle in this did not break down.

←←←←← Centrifuged eggs stained by method II (toluidine blue).

Several of the observations discussed above were used by the Belgian group of Pasteels, Dalcq, and Mulnard to support the idea that the β particles are formed from the α particles or, at least, have something transferred to them from the latter during periods of cleavage. This interpretation was based in part on the following statements:

(1) β particles are not directly stainable, that is, they cannot be stained by merely immersing the egg in staining solutions.

(2) The β particles appear only at times of aster formation when the egg "clears up," that is, when the α particles disappear or decrease in number. In more recent work, however, (Mulnard, 1959; Mulnard *et al.* 1960) it is suggested that β particles may be present in prefertilization stages.

More evidence based on histochemical studies with acid phosphatase and acid mucopolysaccharides is presented by the above group in support of the transfer or assembly idea.

Our observations of β particles contradict the first statement cited above, not only for *Spisula* but also for *Arbacia* (observations by Marsland and Rebhun), which has been worked on by Mulnard *et al.* (1960). In *Spisula* we find β particles present and stainable at all stages from before fertilization, although it may be necessary to stratify the egg to see them clearly and, similarly, we find β particles in fertilized *Arbacia* eggs directly stainable in the stratified egg.

As for the second statement cited above, we attempted to see whether the α particles were, indeed, absent from the egg during the time they seem to disappear or, at least, whether they were present in reduced numbers. To this end we stratified eggs at first cleavage either after or prior to staining. In all cases the results were the same, that is, centripetal and centrifugal layers of metachromatic material were present and little difference in numbers of particles could be discerned between eggs stained in the two different phases relative to centrifugation. The estimation of numbers of particles in stained eggs is, however, grossly inaccurate and highly subjective because of extensive variations from cell to cell and the rapid changes in color intensity in the living eggs. It should be emphasized, however, that during times when α particles appear to be absent from intact eggs, these particles can be centrifuged to the centrifugal pole of the egg by using sufficiently high forces. In addition to the above experiments, we partially stratified eggs by subjecting them to 4300 g for 1 min. at cleavage. In most of these eggs, the stained particles do not separate from the asters, and there are few if any centrifugal or centripetal particles visible, although eggs of the same batch centrifuged longer and, at higher speeds, showed the two layers mentioned previously. We interpret these results and those with methylene blue as follows:

(1) β particles in *Spisula* are present from the unfertilized egg (at least to early blastula stages), are directly stainable, and are attracted to asters whenever the latter are formed (unpublished observations on the activation of egg asters with no subsequent germinal vesicle breakdown support this conclusion, since even here β particles become astrally located; see FIGURE 12).

(2) α particles may be formed at any point in the mitotic cycle, but move into the asters only after pronuclear fusion, and then only if they have been formed prior to the time of the assembly of the aster (since α particles in eggs

stained after aster formation show only an astral localization at the succeeding cleavage).

(3) Our staining results are neutral with respect to the relation between α and β particles postulated by the Belgian group.

With respect to point 3, however, we feel that the electron microscope observations to be presented now lend support to the concept that β particles may be formed from a precursor (although we do not know what relation this precursor has to the α particles); therefore, we must mitigate the criticism we have previously leveled at this concept (Rebhun, 1959b).

Electron microscope studies. Although fixatives with a wide variety of pHs and salt concentrations were used in this study, most of the definitive results came from material that was fixed with Dalton's mixture at pH 7.4 (Dalton *et al.* 1955). The fixative was used either without the addition of salt or with NaCl added to a concentration of 3.1 per cent (approximately isotonic with average sea water). By far the best and most consistent preparations obtained were those embedded in epoxy resins (Araldite 502), although the contrast in Dalton's material embedded in epoxy resins is quite low, and although various staining techniques were required after sectioning (Watson, 1958). There are consistent differences between the fixative used with and without salt, and one can immediately recognize the type of fixation from these differences. In material fixed without added salt the cortical granules appear almost empty with only a small amount of material in them, the chromosomes appear dense, and the outer granular layer of the vitelline membrane is very light, as if material had been extracted. When salt is added, the cortical granules look dense (resembling yolk granules), the outer granular layer of the vitelline membrane (Rebhun, 1958) also looks dense, and the chromosomes so light that they often appear as negative images in the spindle region. Other components of the egg show no consistent differences. Thus lipid granules, yolk, mitochondria, ergastoplasm and, most important, the granules we are about to discuss are indistinguishable in morphology with the two modifications of Dalton's mixture.

In attempting to determine what particles in the electron microscope correspond to the stained particles seen with the light microscope, we made use of a knowledge as to where the particles were to be expected at different times in mitosis and where they go in stratified eggs. Thus eggs, both stratified and unstratified, were fixed at various times from before fertilization to after second cleavage. Our results indicate quite clearly that there are four categories of particles excluded from possible identification with the metachromatic granules. These are lipid, mitochondria, yolk, and cortical granules. In unstratified eggs, the first three categories of inclusions are distributed uniformly in the egg outside the spindle and centrosome region, and in the stratified egg the first two appear in layers not containing the metachromatic particles. The yolk particles that go to the centrifugal hemisphere of the egg, to which the α particles also go, are distinguished very easily from the latter by their much greater size and lack of staining. The cortical granules do occasionally show the astral location in first-cleavage eggs (at least, in the peripheral aster) and uniformly in later cleavages. However, these granules stain orthochromatically: that is,

blue, and not metachromatically: that is, reddish-purple, (FIGURES 8 and 11). They are somewhat elongate in shape, are considerably larger than metachromatic granules, are never present in the asters in as great quantity as the latter, and stratify to a layer centrifugal to the yolk, that is, they are the heaviest formed element in the egg. We shall treat them in detail at a later date.

We have consistently found certain particles, to be described now, in all regions in which we expect to see metachromatic β granules, that is, in aster regions, in the region surrounding the peripheral centrosome of blastomere nuclei, and in the sublipid layer in centrifuged eggs. In sections, the particles are circular to oblong in shape, are from less than one fourth to more than one half μ in diameter or maximum dimension, and are partially or completely surrounded by a single dense layer about 50 Å thick. The interior of the particle contains a number of smaller bodies from 250 Å to more than 500 Å in diameter whose profiles, the walls of which are approximately 50 Å in thickness, are circular, and whose center is transparent to electrons (FIGURES 13, 14, 15, and 29). Some of the smaller vesicles appear relatively solid, probably due to the fact that they may lie completely within the section (FIGURE 16). The number of small vesicles varies in a given cross section but, in particles whose profiles are of about the same outside diameter, one may find anywhere from 3 to 40 of them. In many cases particles may be found that not only contain vesicles inside but have small vesicles adherent to the outside, and the dimensions, shape, and general appearance of which are indistinguishable from the same properties of the inner vesicles (FIGURE 17). In other cases, the external membrane of the particles is incomplete, the ends of the membrane often curling back into the interior of the particle (FIGURES 14 and 15). However, the total region consisting of partial outer layer and inner (and sometimes outer) vesicles appears to possess a morphologic unity. In many cases particles occur in aggregates of from 2 to 6, the group defining a region that often contains, in addition to the larger particles, small vesicles of the type already described and "stray" pieces of "membrane" (FIGURES 14, 18, and 19). Such groups usually contain both particles with incomplete profiles and particles with complete ones. It should be emphasized that metachromatic particles in living cells often form groups similar in number. We have described such "grapelike" masses previously (Rebhun, 1959*b*).

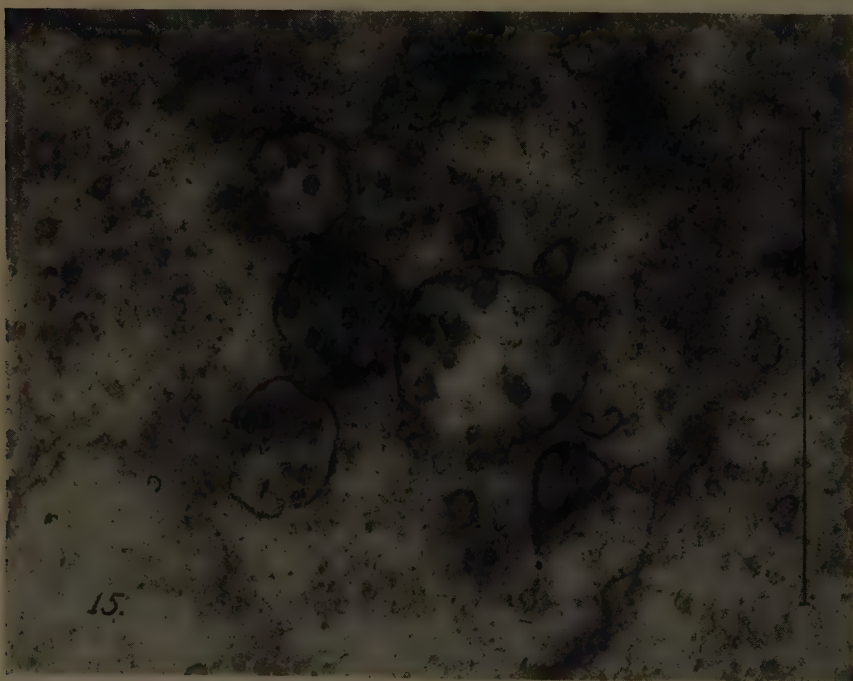
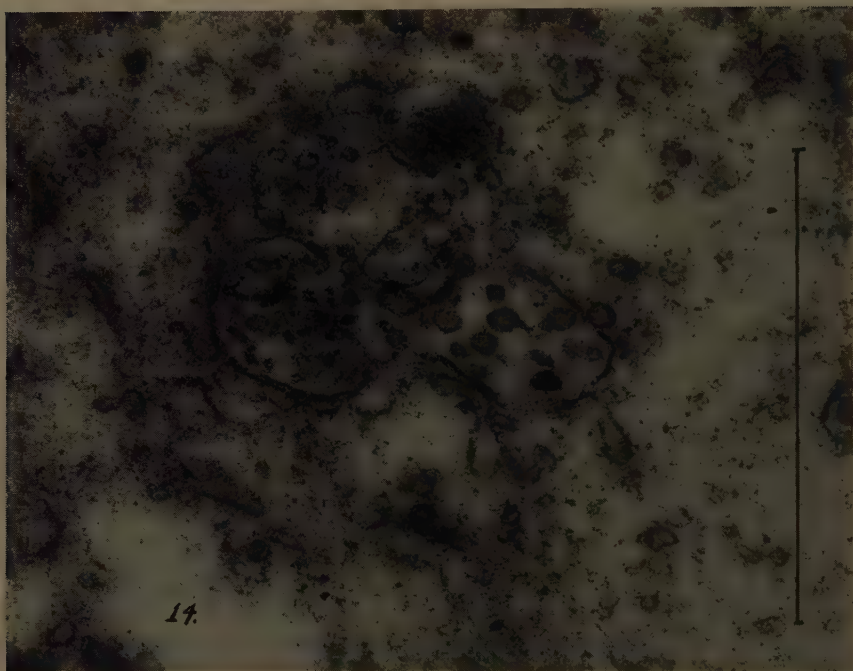
We have identified these particles with the β particles primarily on the basis of size, location in the unstratified egg (coupled with the more uniform localization of other particles previously discussed), formation of aggregates and, most significantly, location in the sublipid layer and association with the asters of centrifuged eggs. The latter requires some expansion.

The hyaline layer (the layer between the lipid and mitochondrial regions) of eggs stratified at about first cleavage actually consists of two layers as seen either with stained eggs or with the electron microscope (FIGURE 11). In stained, living eggs at this stage the metachromatic particles actually are not applied directly to the centrifugal part of the lipid zone, but are separated from the latter by a clear zone approximately 4 to 5 μ thick. Immediately below this layer one finds the stained particles (an occasional one can be found in the upper hyaline layer) usually formed into a thin layer. Occasionally, however, they have a wider distribution in the remaining 15 to 20 μ thick clear

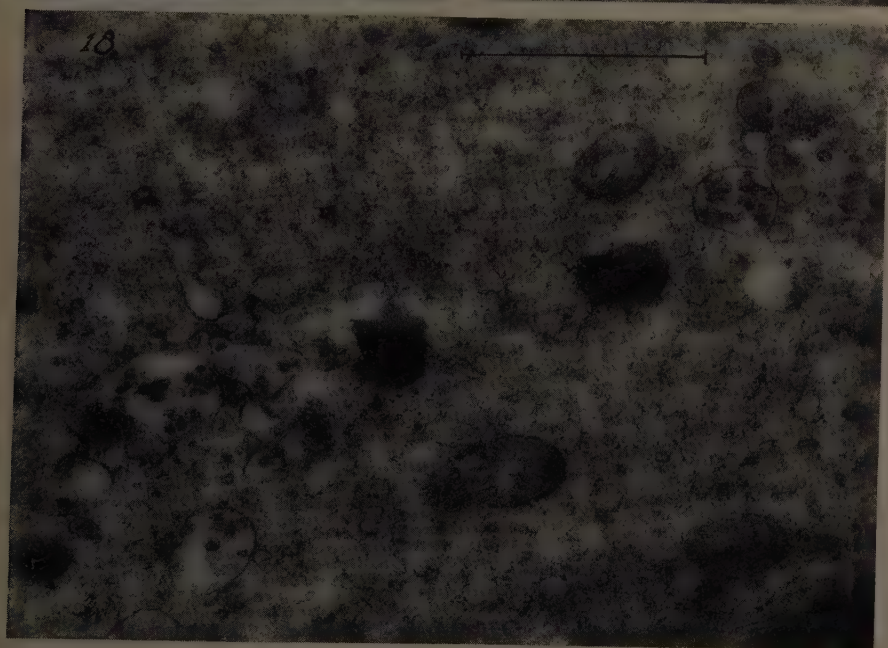
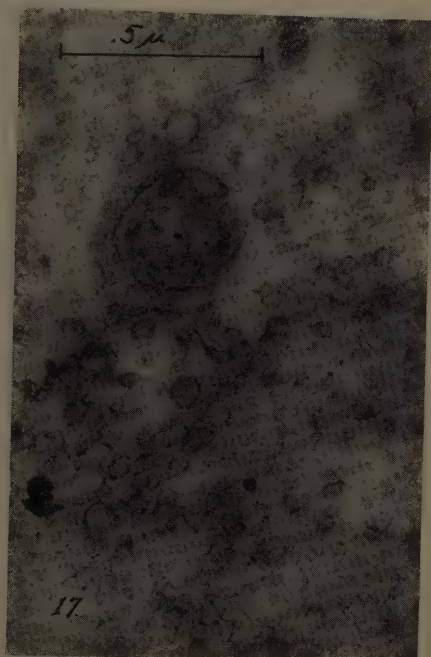
layer between the upper hyaline zone and the mitochondria. When asters can be seen in the egg, they occupy part of this lower zone, and it is usually in connection with the asters that the particles have a wide distribution in the lower hyaline zone.



FIGURE 13. Groups of multivesicular bodies just centrifugal to the upper hyaline zone. Egg stratified just prior to first cleavage.



FIGURES 14, 15. Enlargements of regions of FIGURE 13 showing details of internal and external small vesicles and incomplete membranes.



FIGURES 16, 17. Multivesicular bodies from fertilized eggs stratified just prior to first cleavage. They are located in the upper part of the lower hyaline layer.

FIGURE 18. Multivesicular bodies in the cytoplasm between the nucleus and the cortex in the CD blastomere, between first and second cleavage.

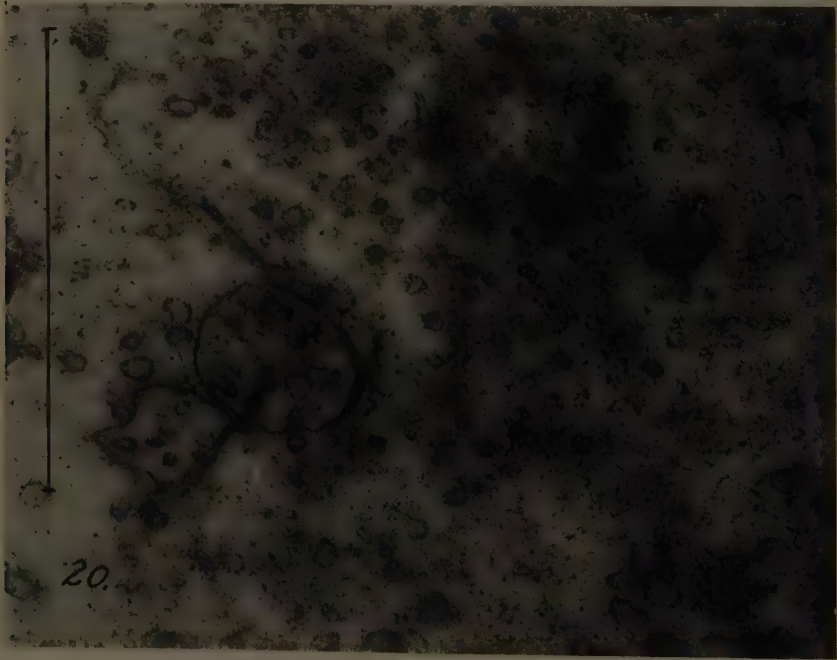
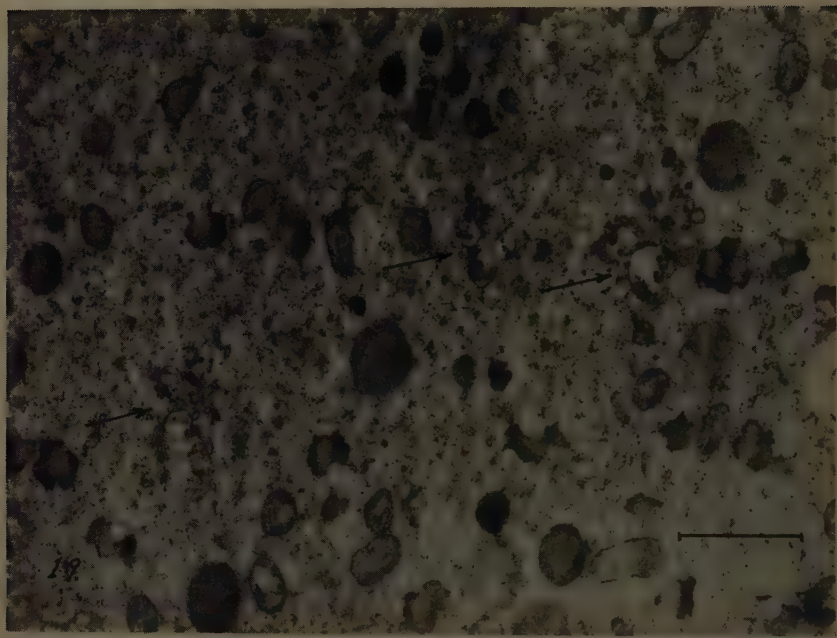


FIGURE 19. Three groups of multivesicular bodies at the arrows. Stage and approximate positions in CD blastomere, as in FIGURE 18.
FIGURE 20. Enlarged region of FIGURE 13. In addition to a multivesicular body a region containing thirty or more small vesicles can be seen.

In the electron microscope, the upper hyaline zone is visibly occupied by large numbers of "rough" and "smooth" surfaced vesicles, although this distinction is not always easily made in this low-contrast epoxy-embedded material. The profiles of the former vary from small (800 to 1000 Å) wrinkled circles to long (3 to 4 μ) loops about 300 to 1000 Å in width (FIGURE 22). These are undoubtedly parts of the ergastoplasm of the egg, perhaps broken to small pieces during centrifugation. The smooth-surfaced vesicles are fewer in number and resemble in many ways (for example in size and shape) the vesicles associated with the β particles. In addition to being devoid of small external granules, the smooth-surfaced profiles are much more regular in shape than the rough-surfaced variety. Finally, they have a tendency to be concentrated into regions up to about 1 μ in diameter and approximately spherical in shape, in which up to 50 or more small vesicles may be found in a given section (FIGURE 20).

The asters in the hyaline zone are characterized by long chains of rough-surfaced vesicles of various lengths in tandem and radiating from a clear region several microns in diameter (FIGURES 21 to 24). From electron micrographs of spindles and centrosomal regions of unstratified eggs in which centrioles can be identified (FIGURE 25) it is apparent that this clear zone (usually, with a denser region immediately surrounding the centriole) is the centrosome. It should be pointed out that in the living egg the asters in the lower hyaline zone may show only astral rays radiating from the portion of the centrosome pointing toward the hyaline zone (that is, not into the yolk region—at least for several minutes after centrifugation) and that, in the electron microscope, this region is the one occupied by the radiating chains of ergastoplasm. It is clear, however, from electron micrographs of nonstratified eggs that this radial arrangement of ergastoplasm is only one component of orientation in the aster and that there appears to be more oriented ergastoplasm in asters in centrifuged eggs than in asters of nonstratified eggs. In favorable electron micrographs, fibers and granules of the spindle and aster appear also to be oriented in long chains, the fibers and granules resembling in detail the same components found in the ground substance (FIGURE 26). Since visible asters often take several minutes to reform in stratified eggs, we feel that the orientation of the ergastoplasm is a visible manifestation of a more fundamental radial organizing influence originating from the centrosome region (influences of this sort have been discussed by Pollister, 1941), which causes elements of the ergastoplasm and, probably, the ground substance to move into the astral regions in streams. Such elements probably then link up in tandem in the chains already discussed. The temporary one-sidedness of the aster in the living cell probably is due to the fact that the ergastoplasm, the orientation of which accentuates the visible astral structure, can be moved into the aster only from the sublipid layer to which it has been centrifuged. Later, the influence of the aster may be seen in the yolk region as a radial orientation of the yolk, although no ergastoplasm appears to be present.

This digression on the aster was made to indicate that the aster can be seen and clearly recognized in the centrifuged egg. Part of the evidence for identifying the multivesicular elements described before with the β particles is the fact that they are closely associated with the asters in living eggs, an association



FIGURE 21. Aster in an egg stratified at first cleavage. Three groups of multivesicular bodies can be seen at the arrows. The lipid layer is seen at the top (L), then the upper hyaline layer (UH), the aster occupies the lower hyaline layer and the mitochondrial layer can be just seen below. The vitelline membrane is marked V.

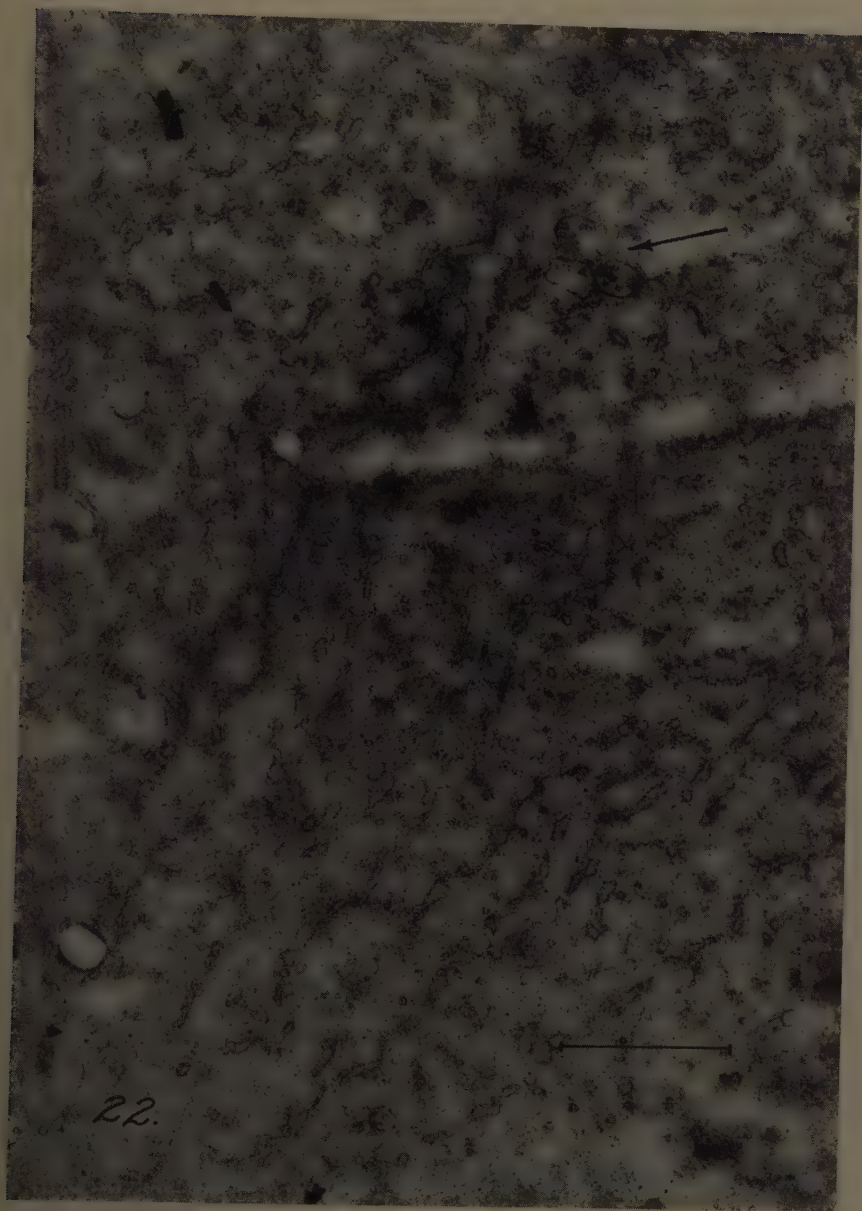


FIGURE 22. An enlarged view of FIGURE 21 showing the chains of rough-surfaced vesicles forming part of the aster and three multivesicular bodies at the arrow.



FIGURE 23. Aster in another cell treated in a fashion similar to the egg in FIGURE 21. Arrow points to a multivesicular body.

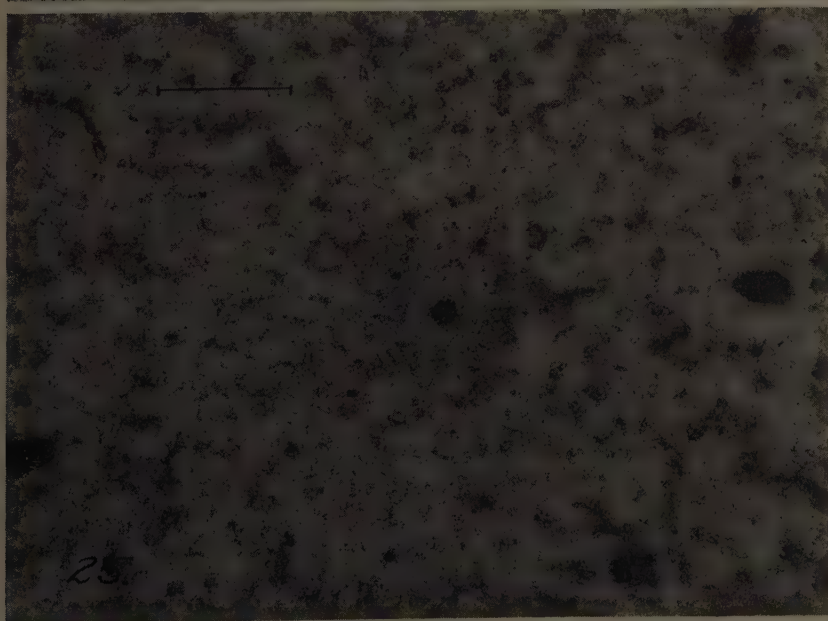
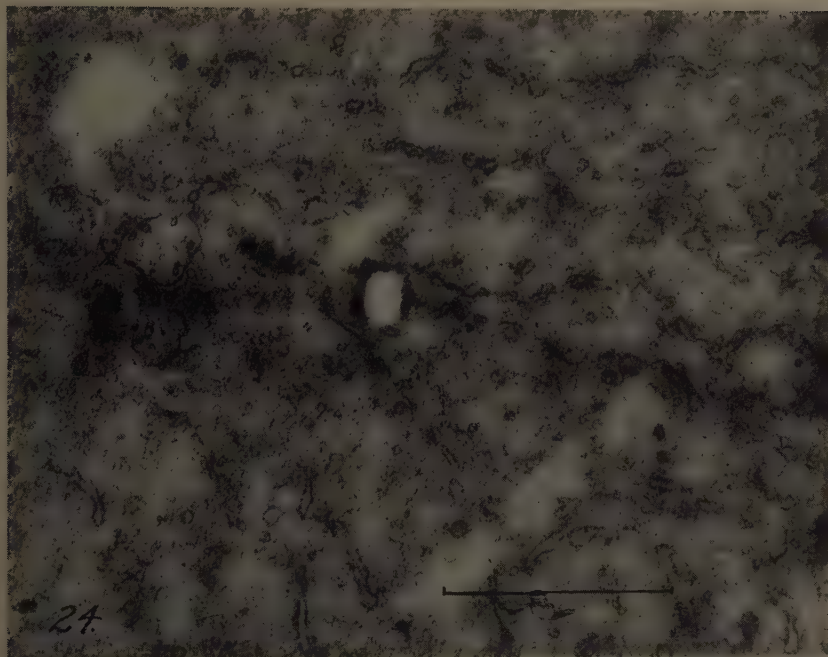


FIGURE 24. Enlargement of part of FIGURE 21 showing considerations similar to those in FIGURE 22. Multivesicular bodies near the aster apex.

FIGURE 25. Centrosome of an egg aster at 11 minutes past fertilization. The nuclear envelope has just fragmented.



FIGURE 26. Peripheral end of a first polar body spindle. Arrows point to regions where linear orientation is apparent. The oriented elements appear similar to unoriented elements in the ground cytoplasm. Ce = centrosome region.

that may be seen clearly also in many electron micrographs of centrifuged eggs (FIGURE 21).

There are other components of the cytoplasm that may be seen occasionally, but more rarely, in fertilized *Spisula* eggs. These are Golgi bodies and annulate lamellae. The former are bodies about $\frac{1}{2} \mu$ thick and 1μ long, in



FIGURE 27. Cytoplasm of an unfertilized egg showing two Golgi regions. Note similarity to vesicle regions in FIGURES 20 and 28. N = nucleus.

profile, consisting of 3 to 6 flattened profiles, with clouds of small 250 to 750 Å smooth vesicles at the ends, similar in detail to bodies described by Afzelius (1956) in sea urchin material. In early *Spisula* oöcytes they are plentiful, but as the egg matures they become very rare in electron micrographs, (FIGURE 27). In centrifuged fertilized eggs we have occasionally seen them in the hyaline zone (either one). The annulate lamellae are bodies about the same over-all size as the Golgi bodies and consist also of 3 to 6 flattened vesicles with the typical annulate lamellar structure (Rebhun, 1956). Vesicles can also be seen at their ends, but in this case they are rough-surfaced and may be in the form of elongate loops. The annulate lamellae are more plentiful than the Golgi bodies and have on occasion been seen to follow the general orientation of the ergastoplasm relative to the asters.

Discussion

I have mentioned earlier the hypothesis of Pasteels, Dalcq, and Mulnard concerning the conversion of α to β particles and have indicated some of the evidence adduced by that group to support this concept (more evidence is presented by them than I have given). A key point, however, is the so-called indirect staining of the β particles, that is, the idea that the latter must get their stain from the α particles, an observation that I have not been able to verify either for *Spisula* (whose egg greatly resembles that of *Barnea*, worked on by the Belgian group) or for *Arbacia* (worked on by Mulnard). Therefore, in a previous publication (Rebhun, 1959b), we have indicated our somewhat acid opinion with respect to this conversion. The electron microscope studies have, however, tended to make us lean more favorably toward the concept of the assembly of β particles from certain precursor elements. The observations underlying this concept are the following:

(1) The β particles as seen in the electron microscope appear to be composite, having an outer envelope and containing small vesicles.

(2) Vesicles of similar form not infrequently are found attached to the exterior surface of the β particles.

(3) The outer membrane of the particles often is incomplete, the ends of the membrane curling *inward*. It should be pointed out that this is true of cells fixed with isotonic solutions as well as of those fixed with hypotonic solutions and thus does not appear to be due to osmotic rupture of the outer layer.

(4) The inner vesicles and adherent outer ones resemble in all respects (size, smoothness, and regular circular outline) other smooth-walled vesicles found in the upper hyaline layer.

(5) Occasionally, spherical aggregates of smooth-walled vesicles may be seen in the cytoplasm, whose total diameter is about 1 μ .

(6) In living stained material the β particles were never observed to break down during cleavage or in later stages even if they had been stained several cleavages earlier.

Statement 6 is evidence against the reverse sequence of events in living eggs, that is, that the small vesicles are liberated by rupture of the β particles.

It is clear that none of the evidence cited is critical for accepting the hypothesis. With respect to the outer adhering vesicles and incomplete membrane, effects other than osmotic ones conceivably could rupture the β -particles

during fixation or subsequent embedding, liberating the vesicles. On the other hand the material, in all other respects, seems to be very gently handled by fixation in isotonic Dalton's fixative and epoxy embedding, and more familiar cytoplasmic elements seem to have suffered little damage. Consequently, we believe the electron micrographs to be fairly accurate representations of the actual structures in the living egg. Finally, despite the reservations we have stated earlier, the idea of the assembly of β particles from precursors is not *contradicted* by the staining results.

The multivesicular bodies described in rat ova by Sotelo and Porter (1959) resemble to a great degree the particles described in this paper. The small interior vesicles, the regions of small vesicles not associated with membranes, the incomplete membranes partially surrounding some of the multivesicular bodies, and the regions of multivesicular bodies and small vesicles are highly reminiscent of the detailed descriptions of β particles given in this paper, although we have not seen evidence of a "nucleoid" as reported in rat ova. Since metachromatic granules in rat ova as described by Dalcq (1954) show some of the phenomena seen in invertebrate eggs (specifically, the aggregation on the peripheral caps of blastomere nuclei), it seems not unreasonable to make the tentative suggestion that the multivesicular bodies in rat ova are the β particles described by Dalcq. This suggestion would appear also to be supported by the statement in Sotelo and Porter (1959) that the multivesicular bodies or small vesicles appear related to the pronuclei and centrosomal regions in later stages of cleavage—these statements were not elaborated on by Sotelo and Porter (1959). The interpretation placed upon the relation between multivesicular bodies and small vesicles by Sotelo and Porter (1959) is just the reverse of ours; that is, they feel that the vesicles are derived by breakdown of the former. Although it is not possible to decide with complete certainty between breakdown or assembly of the particles from the material presented, we feel that the total picture of electron micrographs, plus vital staining, strongly support the idea of assembly of β particles from precursor vesicles as described above.

To speculate further on the process of assembly of the vesicles or on the origins of the hypothesized precursors is clearly premature—but somewhat irresistible. We feel that the hypothesis describing the aggregation of small vesicles and their envelopment by an outer membrane to form β particles is not too far-fetched. Alternatively, these particles could be formed by the fusion of small vesicles with pre-existent large vacuoles, as in Rose's work on microkinetospheres in HeLa cells. With respect to origins of the small vesicles, we have been struck by the resemblance of the vesicles in size, smoothness, shape, and aggregation to the vesicles seen at the ends of the Golgi bodies in the egg (FIGURES 20, 27, and 28). Thus, to complete the round of imagination indulged in above, we suggest as a working hypothesis to tie all the observations together, as follows: (1) the Golgi bodies at various times in the egg's history give rise to aggregates of small vesicles of the type described; and (2) at other points in the cell's life these vesicles become enveloped by membranes in some manner, probably in the regions of grapelike aggregates of β particles described above to themselves from β particles.

Having a new particle to consider, with interesting distribution properties

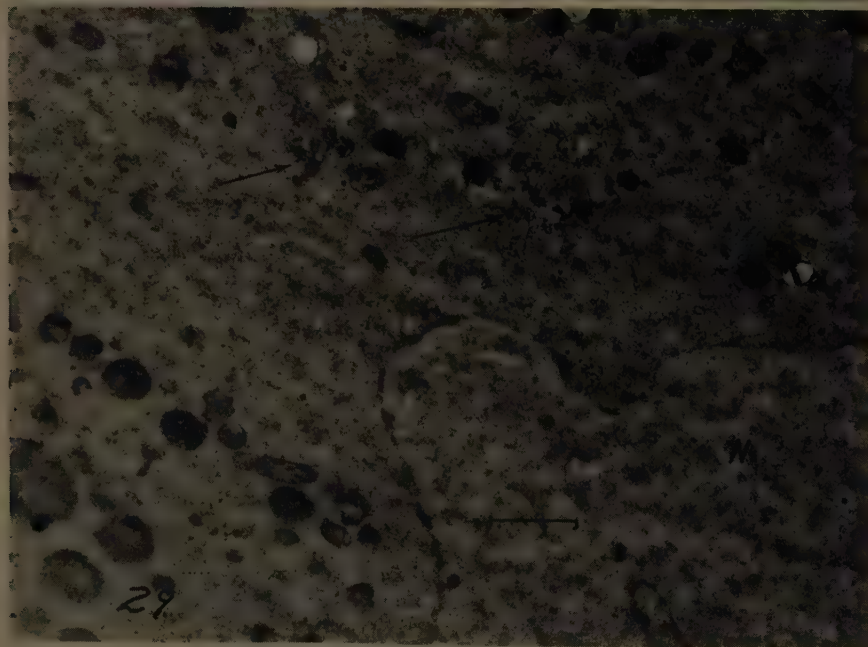
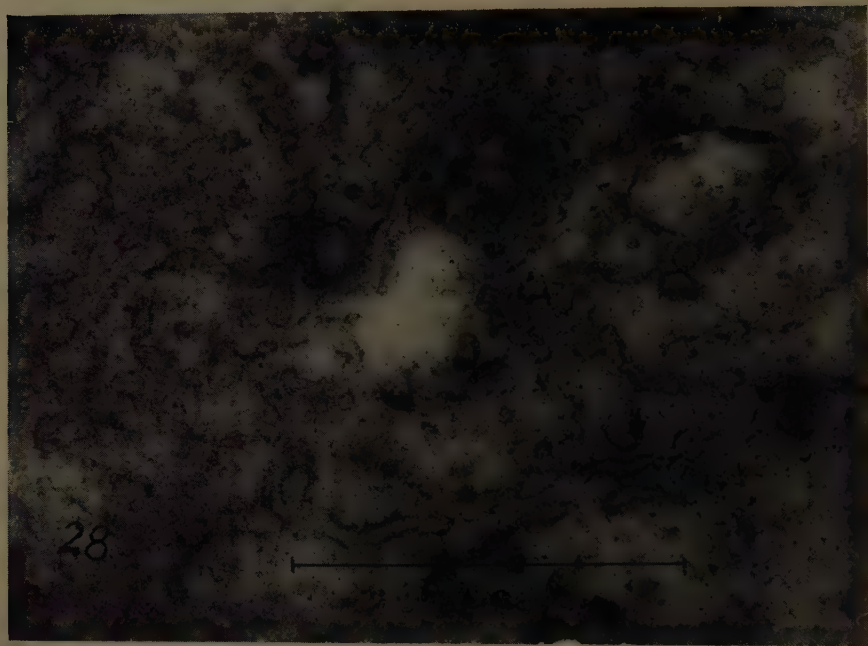


FIGURE 28. Vesiculate regions in upper hyaline zone of an egg stratified at first cleavage.

FIGURE 29. Two groups of multivesicular bodies in an egg between first and second cleavage. N = nucleus.

already described, it is difficult to resist asking for some function. Two have been suggested. The first has to do with differentiation and is based upon the observation that, as cleavage proceeds, not all blastomeres receive the same number of particles, suggesting that some component of the cell is nonuniformly distributed to progeny of the original egg cell by the spindle, resulting in a very early segregation of materials to be used in differentiation. The second suggested function is more solidly based experimentally and has been independently formulated by two investigators: Marsland (elsewhere in this monograph) and Kojima (1959). Both have presented evidence that the particles are in some fashion related to the induction of the cleavage furrow, Marsland's work implicating, in addition, a factor from the nucleus. Since these matters will be presented in more detail in another paper in this publication, we desist from further comments (see A. Zimmerman and D. Marsland, elsewhere in these pages).

Acknowledgment

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AN INTERPRETATION OF TRANSPORT PHENOMENA AT MITOSIS*

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Introduction

During the course of mitosis there are found a number of different movement phenomena and, although the anaphase movements of the chromosomes may appear as the most striking and spectacular event of mitosis, it must be borne in mind that anaphase is a continuation of the previous mitotic stages and that these previous stages also imply important phenomena of chromosomal movements and equilibria, which to a large extent might be due to the same or related mechanisms as also work during anaphase. For this reason, it is always necessary to consider the different mitotic stages together and to pay attention to the possibility that different movements might largely be due to a continued action of the same or related mechanisms.

It should also be noted that, besides the centromere movements that are the most important factor in securing the purposeful completion of mitosis, there also exist other characteristic movements, such as those of pieces of nucleoli and of chromosome arms. Although some of these movements may have no special function to fulfill in the cell, their study might still be of importance for an understanding of the mechanisms working in the spindle, the same mechanisms that may also in one way or another be involved in the centromere movements.

It is with this idea in mind that this paper is devoted not merely to the chromosome movements but also to a number of these other movement phenomena and, for this reason, we are speaking of "transport phenomena at mitosis" in our title instead of merely of the "chromosome movements."

However, this paper is not concerned with all the factors that cause movements of chromosomes and other bodies in the cell during mitosis, but is restricted to consider only the mechanisms that cause movements of bodies relative to the spindle. An idea of the different kinds of movement factors whose influence can be traced during mitosis may be obtained from the following summary of the four different factors that produce the separation of daughter chromosomes during anaphase and telophase.

These factors of the anaphase and telophase separation are:

- (1) A movement of the centromeres on the spindle during anaphase towards the poles.
- (2) A stretching of the spindle during anaphase that separates the poles

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from one another and thus also increases the distance between the daughter groups (Ris, 1949).

(3) In plant mitosis, at least, there is found a pushing effect exerted by the spindle (then called phragmoplast) during telophase on equatorially directed chromosome arms moving them in the two opposite poleward directions and thus decreasing the risk that they may form connections between the two daughter nuclei. This pushing effect is not connected with a spindle stretching (Bajer, 1958*b* and, further, below). The poleward pushing effect acting during late anaphase on certain lagging chromosomes in animal cells, such as the sex chromosomes of *Tipula* and certain Ostracods (Dietz, 1958) might either be due to a related mechanism, or its more specific character may suggest that the mechanism is different, in which case it would represent a factor number (5) in this enumeration.

(4) A strong contraction of the chromosomes at the end of anaphase contributes in pulling equatorially directed arms closer to the polar groups (Bělař, 1929; Bajer, 1959).

In this paper we shall not consider all these factors that contribute in producing the anaphase and telophase separation of the chromosomes but only the factors, Nos. 1 and 3, that imply an actual movement of the chromosomes relative to the spindle. Also, we shall consider certain metaphase and prometaphase effects that appear to be due to the same or related mechanisms as are involved in these two factors. This restriction of the subject matter of the present study should not be taken to mean that we think that the other factors are unimportant, but only that the phenomena considered here form a group of effects that in our opinion may be closely related regarding their mechanical background while, on the other hand, their interrelations with the other effects are much less clear. Naturally, we realize that the anaphase stretching of the spindle also is a mechanism that in many cases to an essential degree contributes in producing the chromosome separation, but its mechanical interrelation with the phenomena in which we are interested here is not sufficiently clear to support a fertile discussion of it in the present connection. The so-called "elimination forces" discussed further below are considered in this article chiefly because they interact with and often strongly modify the expression of the effects in which we are primarily interested here.

The Pulling Force

The movements of the centromeres on the spindle toward the poles during anaphase can be referred to a pulling force acting on them in that direction. The action of a pulling force working on each centromere in the poleward direction is not restricted to anaphase, and there are good reasons for the opinion that forces of this kind influence the centromeres during prometaphase and metaphase (Östergren, 1945*a* and *b*, 1950, 1951; Östergren and Prakken, 1946). This pulling force was called the α -factor of chromosome behavior by Östergren (1949*c*, 1950), and it was described by him as an attraction of the centromere to that spindle pole toward which it is oriented, an attraction of such a kind that the force grows stronger with increased distance between centromere and pole. At ordinary mitosis the centromere unit that behaves in this way is the

chromatid centromere and, as the mitotic metaphase chromosome has two chromatid centromeres that are oriented in opposite directions, this change in intensity of the force with position in the spindle gives the result that the metaphase centromere stabilizes in the equator halfway between the two opposite spindle poles (Östergren, 1945*a*, 1950). After the separation of the chromatid centromeres from one another with the start of anaphase they are free to follow the pulling forces and each of them moves to the pole towards which it is oriented.

It was emphasized by Östergren (1950) that he used the word "attraction" in a purely descriptive way to denote the direction in which the force was working and that it was not his intention to let this word in itself imply any kind of direct interaction between the centromere and the pole. Östergren's interpretation could also very well be described without using the word "attraction," in which case we could say instead that there are "pulling forces acting on the centromeres in the direction toward the spindle poles." Such a change in terminology would not imply any change in the interpretation itself, but it might decrease the risk of certain misunderstandings.

Even the co-orientation of the chromosomes at meiosis, that is, the arrangement of the paired chromosomes that makes it possible for them to separate regularly from one another to opposite spindle poles, can be given a reasonable interpretation in terms of a pulling mechanism acting on the centromeres towards the spindle poles (Östergren, 1951).

In fixed and stained preparations and also in living cells in polarized light it is possible to see a special fibrillar material that is attached to each centromere and extends from it in the direction of that spindle pole toward which the centromere is oriented (see for example the paper by S. Inoué in this monograph). This fibrillar material is the so-called chromosomal spindle fibers. It can scarcely be doubted that these chromosomal fibers are important components of the mechanism producing the pull on the centromeres, but how the force is produced is far from clear.

The centromeres behave in some respects as if they were connected to the spindle poles by means of stretched elastic fibers. There are good reasons, however, to believe that the centromeres are not moved by a simple elastic pull. Thus the chromosomal spindle fibers are of such nature that they permit the body of one chromosome to pass through the traction fibers of another chromosome without interfering with the normal course of mitosis (Östergren, 1949*b*, on the plant *Luzula*). This shows that the traction fibers are far from being stable solid connections between the centromeres and the spindle poles. More evidence for the same opinion is given by observations showing that chromosomes that are attached to traction fibers pulling them toward one pole are still able to turn around in the spindle, to establish such fibers towards the opposite pole and start moving in this opposite direction (Hughes-Schrader, 1943; Östergren, 1951; Dietz, 1956, 1958). Changes of this kind belong to the common and normal events of the meiotic prometaphase.

Since the chromosomal fibers thus appear to be easily reconstructed during the progression of mitosis it is reasonable to suppose, as was done by Östergren (1949*b*), that the changes in length of the chromosomal fibers during the mitotic

movements are also associated with such reconstruction phenomena, that is, that the chromosomal fibers are in a dynamic equilibrium with the rest of the spindle of such kind that these fibers continuously exchange material with the rest of the spindle. According to this idea the length changes of the chromosomal fibers during the mitotic movements would be associated with a loss of material from them to the rest of the spindle when they grow shorter and with a corresponding uptake of material in them when they grow longer.

This idea that the changes in length of the chromosomal fibers are associated with a continuous reconstruction of them of the kind just mentioned is compatible with some different alternative interpretations concerning the function of the chromosomal fibers during mitosis. It was suggested by Östergren (1949b) that the chromosomal fibers represent spindle regions in which the fibriform elementary particles of the spindle are disarranged from their spontaneous equilibrium position by orientation forces acting from the centromeres and that these regions of disarranged particles strive to decrease in size in very much the same way as the surface of a liquid strives to decrease in size by means of surface tension. This was supposed to represent the mechanism by means of which the chromosomal fibers produce their pull on the centromeres.

As this interpretation, however, does not give us a satisfactory explanation of the obvious relations that exist between the movements of the centromeres and certain movements of acentric bodies (see below) we have now reached the opinion that an alternative interpretation may be more probable. This new interpretation is also quite compatible with the opinion that the changes in length of the chromosomal fibers are connected with a continuous reconstruction of them as mentioned above, but in the interpretation it is not assumed that the pulling force is the consequence of an inherent tendency of the chromosomal fibers to decrease their length as far as possible. It is the main purpose of this paper to present this new interpretation.

As emphasized by Dietz (1956, 1958) the pulling force acting on the centromeres changes in intensity from one stage to another. As Dietz points out, the pull is strong in prometaphase and grows weaker toward full metaphase, after which it increases strongly again with the onset of anaphase. According to his opinion the pulling force decreases to zero at the "true metaphase" stage, but in some objects anaphase may interrupt this process before a true metaphase has been reached (Dietz, 1958). Judging from our impression of the chromosome deformations during the meiotic metaphase (mainly in various plant species) it appears very probable that in many materials there is a poleward pull working also at metaphase, and it appears likely to us that the situation with zero force (or something that looks like zero force) at metaphase may be only a rare exception. To call only such special cases "true metaphases" would imply a definition of the metaphase stage that is different from what is currently used, and such a change of terminology would scarcely be very practicable. In any case we agree with Dietz that the poleward pull is strong during prometaphase, that it grows weaker during metaphase, and that it increases again with the onset of anaphase. This may imply that a new mechanism causing the pull may start to operate only with the onset of anaphase,

as suggested by Dietz, or it may imply that the same pulling mechanism that worked before is then passing over into more vigorous action.

There are also other phenomena suggesting that important changes take place in the mitotic mechanism with the onset of anaphase, especially the shifting of the chromosomes toward the poles in cases of mitosis with undivided chromosomes. These chromosomes that consist of single chromatids show a tendency towards equatorial arrangement during metaphase but are later shifted without division toward the poles in a kind of anaphase (Beadle, 1931, on "polymitosis" in maize; Bajer and Östergren, manuscript in preparation, on *Haemanthus*). It is very probable that this change in behavior is connected with the mechanism causing the intensification of the pulling force in normal anaphase, but the nature of this effect is at present far from understood.

Other chromosome regions besides the centromeres can also be found to behave like centromeres on the spindle. These instances of a so-called neocentric activity are in some cases long lasting and dependent on a specific genotypic constitution, in which cases the effect is restricted to meiosis, as in inbred rye (Prakken and Müntzing, 1942; Östergren and Prakken, 1946) or as in maize with an abnormal chromosome 10 (Rhoades and Vilkomerson, 1942; Rhoades, 1952). In other cases the activity is a very short lasting one; in these cases the effect has been observed in ordinary mitotic divisions (studies on living cells of various higher plants, Östergren and Bajer, 1960; Bajer and Östergren, manuscript in preparation) where it acts for a very short fraction of the mitotic period only. In maize and rye the chromosome regions showing this special kind of activity were found to be attached to chromosomal spindle fibers similar to those found at the centromeres (Östergren and Prakken, 1946; Rhoades, 1952). It is quite possible that an attachment of this kind could be a general characteristic of the neocentric chromosome regions and that this presence of chromosomal fibers on these regions might explain why these regions move like centromeres. The movement characteristics of such neocentric chromosome regions are quite different from those of bodies that are not attached to the spindle (acentric bodies, discussed in next paragraph). Still, it is our opinion that the mechanism causing these different types of movement could to a large extent be the same.

An Interpretation of the Mitotic Movements

Centromeres and chromosome regions showing neocentric activity are not the only bodies or chromosome regions that show regular and characteristic movements in the mitotic spindle. It has been found that bodies that are not attached to traction fibers also perform characteristic movements. Although the movement regularities of such acentric bodies are different from those of the centromeres, it is still possible to trace a distinct relationship between these different types of movement behavior, a relationship suggesting that these movements may result from the same fundamental mechanism. These movements of acentric bodies and their relations to the centromere movements form the basis for the theoretical interpretations presented in this paper.

Such "acentric bodies," the movements of which it is possible to study, are pieces of nucleoli that have persisted into prometaphase and metaphase, vari-

ous small granules of unknown and probably variable nature and finally chromosome fragments without centromere (acentric fragments). In plant cells such bodies move to the poles during prometaphase and metaphase. After their poleward movement they may remain either in the polar regions of the spindle or pass into the cytoplasm. Larger bodies such as nucleolar fragments and acentric chromosome fragments lie more stationary when they are in the polar spindle regions, but the small granules jump about irregularly in these polar regions in movements that, at least superficially, look like Brownian movements. The general character of the movements of acentric bodies is schematically represented in the diagrams of FIGURE 1.

We have studied the movements of such acentric bodies in living cells of the endosperm of various plant species, especially in *Haemanthus katharinae* and *Leucojum aestivum*, both in untreated cells and in cells irradiated for the production of acentric fragments (Bajer and Molè-Bajer, 1956; Bajer, 1958*b*; Molè-Bajer and Östergren, in preparation). The poleward movement of persisting nucleoli in plant cells is well known already from many studies on fixed material and is described in numerous papers. In some cases it was even reported that the nucleoli could divide through constriction, after which the pieces were found to separate towards opposite poles, as described in the diagram of FIGURE 1*c*.

The mechanism causing these movements also works on the chromosome arms of long flexible chromosomes and causes them to straighten and to become oriented from the equatorially arranged centromeres in the direction towards the spindle poles, as indicated in FIGURE 1*d*. This arrangement of the chromosome arms was mentioned in passing by Östergren (1949*a*) and described in more detail by Bajer and Molè-Bajer (1956).

Although the poleward movements of the acentric bodies are the dominating feature in their behavior, some of them are also found to leave the spindle in a more lateral direction, an effect that may be ascribed to the influence of the so-called "elimination forces" described below. The influence of these elimination forces on the arrangement of the arms and the difference in arrangement between long flexible chromosomes and more contracted chromosomes will also be considered further, below. The behavior of small granules is more variable and irregular than that of the larger bodies, a difference that in part is likely to be real and in part may be due to technical difficulties of distinguishing between granules inside the spindle and on the spindle surface in flattened endosperm cells.

These poleward movements of acentric bodies take place with the same velocity whether the bodies are small or large (Molè-Bajer and Östergren, in preparation). While observing these movements one might at first get the impression that the acentric bodies were carried by a kind of current of liquid spindle material streaming in the poleward direction.

A more detailed study, however, does not support the interpretation that these bodies simply lie in a liquid that is streaming as a whole toward the poles. This is especially clear from a methanol-treated endosperm cell of *Haemanthus* that will be described elsewhere (Molè-Bajer and Östergren, in preparation). In cells poisoned with methanol there are often found disturbances of the

spindle polarity that are expressed by the occurrence of multipolar mitotic figures. Although the disturbance in the present cell was of another kind it might still be attributed to a disturbed spindle polarity. In this cell we had a spindle pole lying inside one of the half spindles. (We may rather consider

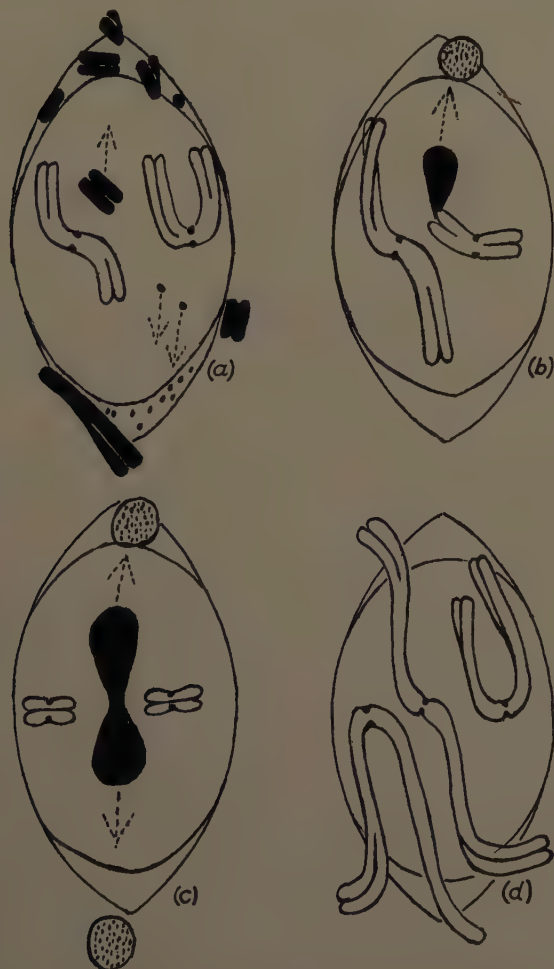


FIGURE 1. Diagrams showing the principles of movement of acentric bodies such as they appear at mitosis in higher plants. Centric chromosomes are drawn in outline, while acentric bodies (chromosome fragments, nucleoli, and small granules) are drawn black. The direction of movement is indicated by arrows, and the final position of the nucleoli is shown by the same bodies drawn dotted. (a) During prometaphase and early metaphase acentric fragments and small granules move in the poleward directions and are deposited in the polar spindle regions or pass into the cytoplasm. (b and c) Nucleoli persisting in prometaphase and metaphase often take a droplike (b) or dumb-bell (c) shape and move to the polar spindle region or into the cytoplasm undivided, as in b, or after division into two, as in c. (d) The arms of long and thin chromosomes are arranged in such a way that they extend from the equatorially located centromeres in the direction toward (and often beyond) the spindle poles. Where the arms leave the spindle to extend into the cytoplasm they are often bent in various directions.

the figure in question as a kind of aster.) In this case numerous small granules were found to accumulate at this pole by moving to it from all other directions (FIGURE 2a). One does not expect a cytoplasmic current to be able to produce this kind of effect. A current does not stop in one place like this; it would have to continue and to carry the granules along with it. For this reason we think that the granules (and the other acentric bodies) are carried by a special transporting mechanism working in the spindle substance, while the spindle substance itself remains stationary. We think that it might be suitable to call this kind of motile influence a pumping activity of the spindle, because in the case of a pump we have machinery that transports material, while the machinery itself remains in one place.

This pumping activity is schematically represented in the diagram of FIGURE 2b. During metaphase it works from the equator toward the two opposite spindle poles. It is emphasized that we do not assume a kind of pumping producing currents of liquid, but only a pumping activity working directly on bodies included in the spindle and causing them to move without the intermediation of a liquid current. FIGURE 2b should not be taken to illustrate the flow of currents but is a diagram drawn to show the direction of a pumping activity by means of which a stationary spindle substance works on included bodies.

A symbolic representation of the pumping mechanism is given in FIGURE 3. The spindle is supposed to contain thousands or millions of units of macromolecular dimensions pumping in the same direction. These have been represented here by small rectangles each with four wheels. We emphasize the purely symbolic nature of this representation. Thus we do not intend to suggest that the molecular pumps contain real wheels. These pumps or motive elements (if we prefer to call them so) cause a movement of included bodies, but as they pump in the same direction they do not exert a moving influence on one another.

The motive elements cannot be supposed to lie quite free from one another, as they are depicted in FIGURE 3, but they must be connected mutually by some sort of links or forces that give the whole system a sufficient rigidity that makes it possible for the spindle to carry the load of the forces by means of which it moves included bodies. On the other hand, these links that connect the spindle components to one another cannot be too firm and stable as these components must have a certain mobility relative to one another to permit the movements of bodies inside the spindle. It is possible that easily reversible links between the spindle components, links that are continuously being dissolved and reconstructed, may explain the two seemingly contradictory properties of the spindle: rigidity and fluidity, permitting the movements of bodies inside it (Östergren and Prakken, 1946).

One may wonder what the real nature of the "pumping mechanism" might be. At present, only loose suppositions may be expressed in this respect, and it is outside the scope of this paper to express any definite opinions in this respect. One possibility that might be considered is expressed in the idea that there may be wave movements passing along fibrous structures within the spindle. Although the fibers themselves do not move in the longitudinal di-

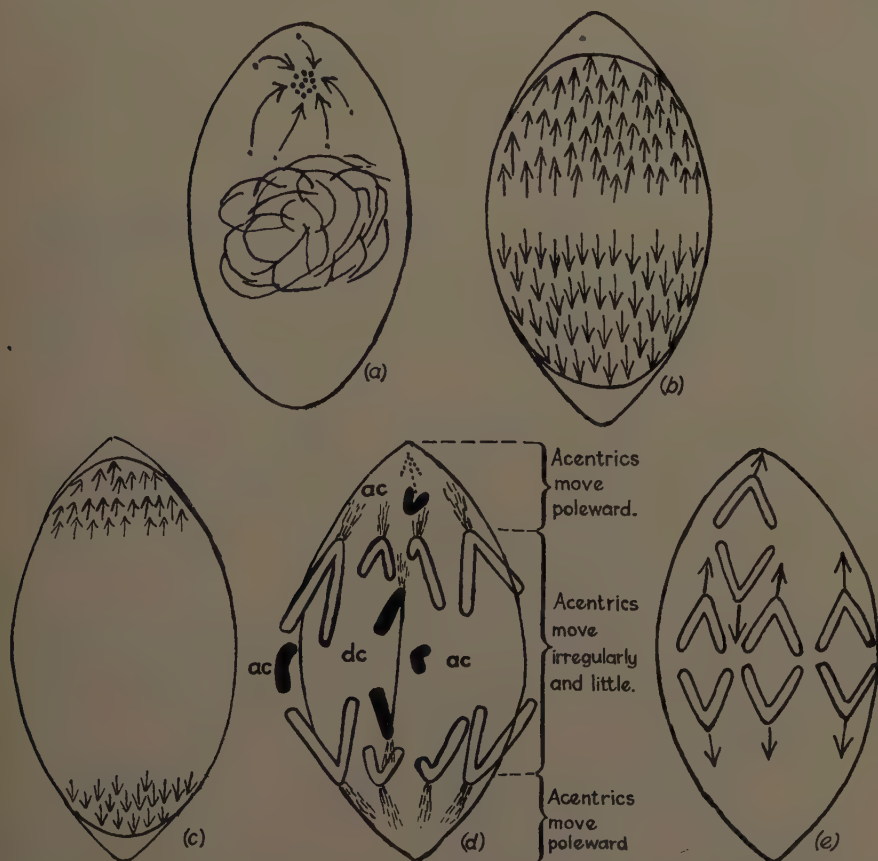


FIGURE 2. The pumping activity and its dependence on the centromeres and their traction fibers. (a) Diagram of a methanol-treated cell from the study of Molè-Bajer and Östergren where the small granules move toward and accumulate in the polar region from all around it, a behavior giving evidence against simple currents as the motive mechanism. (b) A schematic representation of the pumping activity working at metaphase from the spindle equator in the two opposite poleward directions. In the most equatorial region there is likely to be a dead zone without pumping activity. (c) During anaphase the pumping activity disappears from the interzonal region and remains in the half spindles only, a fact suggesting that it is provoked by the centromeres probably through the mediation of their traction fibers. (d) A diagram of the movement characteristics of acentric (*ac*) and dicentric (*dc*) chromosomes during anaphase. These are drawn black; the monocentric ones in outline. In the half spindles the acentrics move poleward (arrow) but in the interzonal region they move irregularly and but little. The free arms of dicentric bridges are equatorially directed and thus cannot be subjected to a poleward pumping mechanism. The pumping activity corresponding to this pattern of behavior is represented in *c*. (e) When a chromosome lying outside the metaphase plate passes into anaphase, one of its daughter chromosomes will meet other anaphase chromosomes moving close to it in the opposite direction. On the basis of the interpretation (see FIGURE 4a) that the centromere movements are due to the pumping activity working at their traction fibers, the situation in a case such as that of (e) can mean only that the direction of the pumping activity is determined by the individual traction fiber to be away from its centromere. The traction fiber should be surrounded here by a streak of spindle material where the pumping works in a direction opposite to that in the neighboring spindle regions.

rection of the spindle, there could be wave motions passing along these fibers in the longitudinal direction toward the spindle poles. This would imply a mechanism similar to that of the peristaltic movements of the intestine. By means of these movements the intestine transports bodies inside it without moving in the longitudinal direction itself.

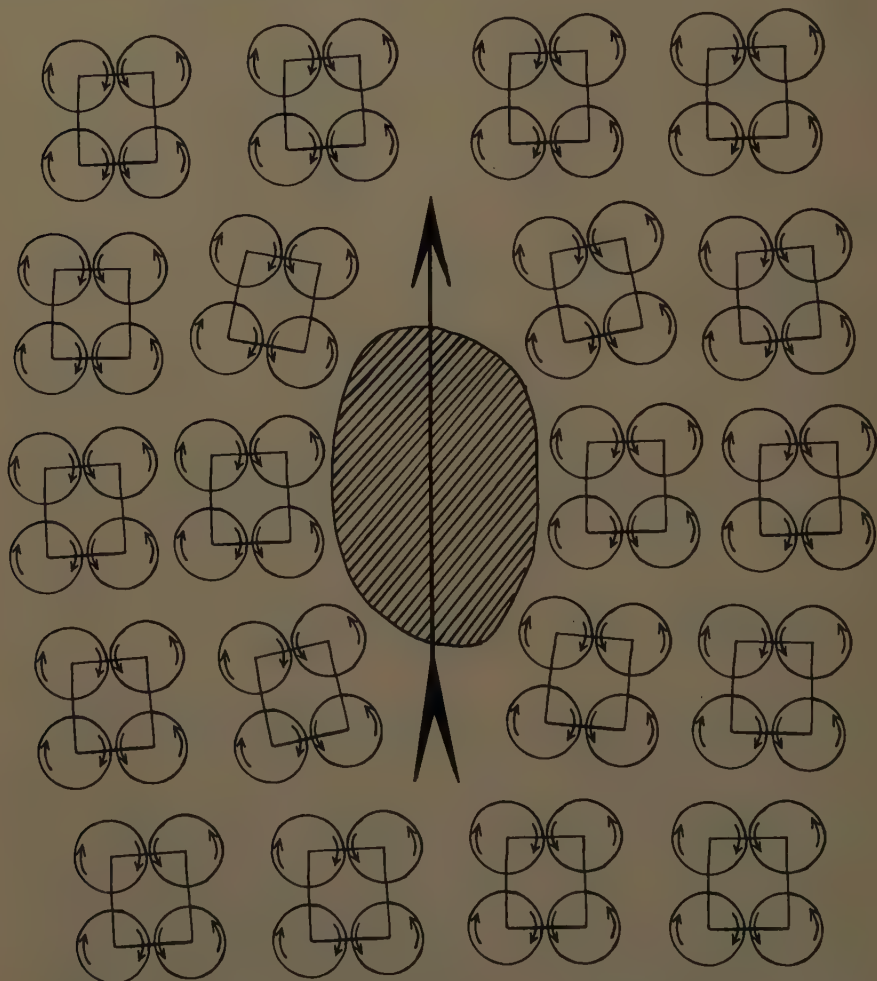


FIGURE 3. A symbolic representation of the mechanism of the pumping activity in the spindle. The spindle is supposed to contain thousands or millions of motive elements of macromolecular dimensions that, over large regions of the spindle, pump in the same direction and in this way cause acentric bodies (such as the cross-hatched granule in the figure) to move. Only a very small part of the spindle is represented in the figure.

We emphasize that it is not our intention to imply the concept of a rhythmic action in the expression "pumping activity." In our opinion the motive elements working inside the spindle may conceivably work in a rhythmical way, but they might also quite conceivably have a continuous manner of action, and

we do not feel justified in committing ourselves to either of these two alternatives.

Special reference has been made above to a cell that was poisoned with methanol and to the movements of small granules within this cell. It should be mentioned, however, that the present interpretation is well supported also by the movements of acentric bodies in general in untreated* cells. Thus it is found that acentric fragments move to the polar spindle regions where the movement often stops and the fragments accumulate. This accumulation is not compatible with an interpretation that the fragments are carried by a simple current of streaming liquid. Many of the fragments, however, are also found to pass into the cytoplasm and are then carried about by cytoplasmic currents to various other positions in the cell. Bajer (1958*b*) reports that the spindle stretching in anaphase exerts a piston action on the cytoplasm, causing it to flow in the equatorial direction carrying the fragments with it.

It has been pointed out above that the spindle to a certain degree has a liquid character permitting the movements of bodies within it. It is even possible for the body of one chromosome to pass transversely through the traction fibers of another chromosome without interfering with the normal course of mitosis (Östergren, 1949*b*). Under these circumstances it would be only natural to suppose that there would occur also some flow phenomena within the spindle. The present interpretation does not imply that there does not occur flow motions of the spindle substance. It is quite possible that such movements may also occur. The essential point of our interpretation is merely that such streamings within the spindle are not the predominant mechanism causing the movement regularities of acentric bodies.

One more argument against the idea of simple currents as the cause of the effects discussed here is to be found in the arrangement of chromosome arms in the spindle equator. If there had been poleward currents of the spindle substance in both half spindles during metaphase, then it would be expected that the material that streams towards the poles from the equator would enter the equator laterally from the sides of the spindle. The arrangement of chromosome arms in the equator, however, gives no evidence for such currents directed toward the center of the metaphase plate. On the contrary, the arm arrangement is just the opposite from what would be expected on the basis of such an interpretation in terms of currents (see below concerning the effect of the so-called "elimination forces").

The pumping activity working in the two opposite poleward directions at metaphase (FIGURE 2*b*) very probably has a kind of dead zone in the spindle equator as indicated in the diagram. In this region where there is no poleward pumping there would be a viscous resistance toward the movement of the proximal regions of the chromosome arms in the beginning of anaphase, an effect that could be an essential factor in causing the turning around of the chromosome arms during the anaphase movement.

The velocity of the acentric bodies that move in the spindle toward the poles

* The word "untreated" in this connection refers simply to the fact that the cells had not been treated with poisons changing the spindle function. The acentric fragments had been produced by means of treatment with β -rays (Bajer, 1958*b*), but it cannot be assumed with any probability that this treatment would have changed the way of action of the spindle in the respects in which we are interested here.

during prometaphase is of about the same size as that of the centromere movements during anaphase (Molè-Bajer and Östergren, in preparation). In the more rare cases when acentric bodies can be studied as they move in the half-spindles during anaphase, they also show the same speed as the centromeres. However, in spite of this similarity in the speed of their movements, it is found that the forces acting on centromeres and on acentric bodies are very different in size when a resistance prevents their movements. When the movement is arrested or prevented by a mechanical resistance, it is seen from the deformation of the chromosomes that the forces acting on centromeres are very much stronger than those acting on acentric bodies. Evidence for the strength of the centromere forces is given by the stretching of dicentric anaphase bridges and by the stretching of the intercentromeric regions in bivalents at first metaphase. Evidence for the much weaker action of the forces on acentric bodies is given by the absence of any comparable stretching of chromosome arms that are pumped in the poleward direction from equatorially arranged centromeres in cells such as in FIGURE 1*d*.

In this connection it should be pointed out that the forces acting on centromeres of freely moving anaphase chromosomes must be very much weaker than those acting on centromeres whose movements have been arrested by dicentric bridges. This important difference, whose significance was emphasized by Cornman (1944), is clearly demonstrated by an absence in the freely moving chromosomes of any deformation similar to that found in the bridges. This difference demonstrates that the speed of the chromosome movements in normal anaphase is not conditioned by the size of the equilibrium force that the pulling mechanism is producing when it is outbalanced by a corresponding opposing force. For this reason the speed of the anaphase movements of chromosomes cannot be used to measure or to judge the change in intensity of the equilibrium force that will act on a "braked" centromere with differences in position of this centromere within the spindle. This point is well illustrated by the fact that acentric bodies and centromeres move with about the same speed, although the equilibrium forces that are found when the movements are braked are very different in size.

It seems very unlikely that these movements of acentric bodies in the spindle during mitosis are independent of the mechanism causing the centromere movements. On the contrary, it seems likely that the acentric movements are a by-product of the same mechanism, whose primary function is the moving of centromeres. We suppose that this pumping activity in the spindle that moves the acentric bodies also causes the movements of the centromeres and that it does so by pumping at the chromosomal spindle fibers (FIGURE 4). These chromosomal fibers are likely to consist of numerous very fine fibrillae that spread in the half spindles and offer a large surface upon which the pumping activity may work. This large surface of the chromosomal spindle fibers would explain why the pulling force working at the centromeres when their movement is braked is much larger than the force with which acentric bodies are "pumped" in the poleward direction. The surface on which the pumping activity works is larger in the case of the chromosomal fibers than it is in the case of chromosome arms and acentric fragments. This interpretation also makes it understandable that the speed of the movement in both cases is the

same. There is presumably very little resistance in the spindle to the very slow movements with which we are concerned, and both centromeres and acentric bodies simply move with the speed with which the pumping mechanism is working.

According to this interpretation the chromosomes are propelled in very much the same way as sailboats. The pumping activity is the wind, and the chromosomal spindle fibers are the sails of the chromosomes. This concept would also explain why a longer chromosomal fiber produces a stronger pull than a shorter one, a situation that we have in prometaphase, when the chromosomes

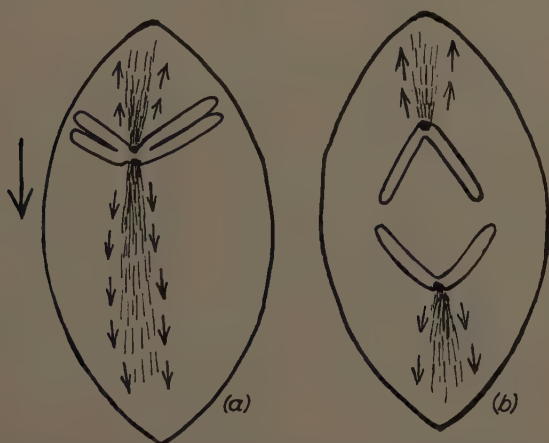


FIGURE 4. The centric chromosomes are moved by the pumping activity working at the traction fibers of their centromeres. These fibers consist of numerous very fine fibrillae and have thus a very large surface. The traction fibers act as sails of the chromosomes. They catch the wind of the pumping activity that they themselves provoke in the spindle. The pumping activity is determined to work in the direction away from each centromere toward that pole toward which the traction fiber is directed, and the traction fiber has been organized in the direction toward that pole toward which the chromatid centromere in question is oriented. The traction fiber can be reconstructed during the reorientations of the centromeres (see text), and it is most probably reconstructed during its shortening and lengthening at the chromosome movements. As a longer traction fiber catches more of the wind of the pumping activity, the metaphase centromere will be pulled by a stronger force toward the more remote pole, an effect causing the metaphase stabilization in the equatorial plate, as shown in *a*. (*a*) Prometaphase. The arrow on the left side of the spindle shows the direction of the chromosome movement. (*b*) Anaphase.

move on to the plate (FIGURE 4a). A larger sail catches more of the wind and a stronger force is obtained. The stronger force on the longer traction fiber explains why the chromosome moves to the plate in prometaphase and is stabilized there during metaphase. In order to make this idea work, it is, however, necessary to make two more assumptions. These two assumptions are not loose constructions made merely to support the present interpretation, but there are also other results that justify these assumptions.

The first of these two points is the opinion that the chromosomal spindle fibers are subjected to continuous reconstruction during the course of mitosis. According to this idea the chromosomal fibers, which are organized by the centromeres toward the spindle poles, grow out from the centromeres until

they reach the poles or until they reach a certain distance from the poles. When the chromosomes move these chromosomal fibers are reconstructed during the movements, that is, they actually grow longer and are not merely stretched when a chromosome moves away from a pole, and they grow shorter through reconstruction when a chromosome approaches a pole. This interpretation is justified by the observations showing that the body of one chromosome can pass through the traction fibers of another and by the fact that centromeres are able to turn around in the spindle in spite of being attached to traction fibers (see above). If this opinion is valid, we shall, of course, really have a larger "sail" toward the more remote pole capable of giving a stronger force in that direction, but the "sails" are reconstructed and have been changed to the same size when a chromosome is stabilized in the equator.

It is commonly assumed that the chromosomes move toward the poles because the chromosomal fibers grow shorter or "contract." According to the present interpretation, however, the causality would be reversed, that is, the traction fibers would grow shorter through reconstruction because the chromosomes move closer to the poles.

The second point to which reference was made above is expressed in the opinion that this pumping activity (by means of which the general background material of the spindle is working on acentric bodies as well as on the traction fibers of the chromosomes) is actually provoked in the spindle by the centromeres (probably through the intermediation of their traction fibers), and that the centromeres also determine the direction of this pumping activity in such a way that it is always directed away from the centromere and toward that spindle pole toward which the traction fiber is oriented. It is not assumed that the spindle is simply divided into two halves by the equator and that there is a pumping activity in these two halves that consistently goes in the two opposite poleward directions. The reason why the pumping activity of the spindle shows this pattern in metaphase (FIGURE 2*b*) is because all the centromeres are stabilized in the equator during this stage. The pumping activity in the background material of the spindle is provoked by the individual traction fibers in those spindle regions that are immediately adjacent to each traction fiber. When the centromeres are not in the equator but spread out over the spindle, as in prometaphase, there will be streaks within the spindle where the pumping activity is directed from the centromeres that are outside the equator across the equatorial region and towards the more remote pole (as in FIGURE 4*a*). Such streaks should sometimes be found lying side-by-side in which the pumping action is directed in the two opposite poleward directions. This would explain why chromosomes can be found moving closely side-by-side in the two opposite poleward directions. Observations of such movements were made by Östergren and Bajer (1960) on endosperm cells of *Haemathus* that had been poisoned by treatment with methanol. In some of these cells chromosomes were found lying far outside the equator during metaphase and, when these chromosomes pass into anaphase, one of their daughter chromosomes was observed to meet the daughter chromosomes coming from the equator (as shown in the diagram of FIGURE 2*e*).

The behavior of acentric fragments and of small granules during anaphase

strongly supports this idea that the centromeres and their traction fibers actually provoke the pumping activity. These acentric bodies make regular poleward movements only in the half spindles, that is, in the regions extending from each centromere group to the corresponding pole, where the traction fibers of the centromeres are located (FIGURE 2d). In the interzonal regions, that is, between the separating centromeres, the acentric bodies move only irregularly and but little (Bajer and Molè-Bajer, 1956; Bajer, 1958*b*; Molè-Bajer and Östergren, in preparation). Thus the pattern of the pumping activity in the spindle during anaphase would correspond to that drawn in the diagram of FIGURE 2c (compare with the metaphase pattern in FIGURE 2b).

This absence of a pumping activity in the interzonal region is also demonstrated by the arrangement normally found during anaphase of chromosome arms that are attached to dicentric bridges. These arms are usually hanging down in the equatorial direction from the centromeres carrying them (also drawn in FIGURE 2d). This arrangement of the free arms carried by bridge centromeres is also evidence that there is no pushing effect from a so-called *Stemmkörper* pushing the chromosomes from behind toward the poles at this stage. The spindle stretching found at this stage does not act by pushing the chromosomes from behind, but it acts by increasing the distance between the poles, as emphasized by Ris (1949). This arrangement of the arms can be seen in many figures in the literature and it has been studied in preparations of fixed material from chromosome breakage experiments by one of us (G. Östergren). At a somewhat later stage, however, we have a pushing effect working toward the poles; but at this stage the pushing effect is not connected with a spindle stretching (Bajer, 1958*b*).

Thus the centromeres with their traction fibers differ in an essential way from the acentric bodies as regards the manner in which they interact with the spindle. Such bodies as acentric fragments and persisting nucleoli are merely carried passively by the pumping activity of the spindle, but the centromeres with their traction fibers actually provoke this pumping activity and determine its direction. Chromosome regions showing neocentric activity obviously interact with the spindle in the same way as centromeres. Neocentric chromosome regions have been shown in the case of *Secale cereale* (Östergren and Prakken, 1946) and *Zea mays* (Rhoades, 1952) to organize chromosomal spindle fibers similar to those found at the centromeres and, as these neocentromeres can be found to be pulled not merely toward the nearest pole but also sometimes toward the more remote one (Östergren and Prakken, 1946; Rhoades 1952), the observations support the interpretation that these chromosomal fibers likewise determine the direction of the pumping activity around them.

The chromosomal spindle fiber is an unusual kind of a sail. It does not merely catch the wind that is there, but it actually provokes the wind and determines the direction of the wind.

The active participation of the centromere (or neocentric region) with its traction fiber in provoking the pumping activity is also suggested by the fact that the size of the pulling force and the speed of the movement of the centromeres change from one stage to another of their active period. As emphasized by Dietz (1956, 1958) the centromeres show rapid and vigorous movements

when they have just organized their traction fiber (an effect corresponding to the prometaphase stretch of Hughes-Schrader, 1943) but the forces grow weaker toward the end of metaphase, after which they increase in size again just before or during the start of anaphase. The very rapid and vigorous movements of the neocentric chromosome regions in the endosperm mitoses of *Haemanthus* (Östergren and Bajer, 1960; Bajer and Östergren, in preparation) may be due to their having a freshly organized chromosomal fiber and that their situation thus in this respect corresponds rather to that of the centromeres during the prometaphase stretch.

Also in the endosperm mitoses of *Haemanthus* the centromeres show especially rapid and vigorous movements when they have just been attached to the spindle, a phenomenon corresponding to the prometaphase stretch (Bajer,



FIGURE 5. In the phragmoplast at telophase the pumping activity (arrows) starts working again. It moves small granules, acentric fragments and chromosome arms in the poleward directions. In the equator of the phragmoplast small granules appear that are to form the new cell wall.

1958a). This period is of short duration for each individual centromere and does not take place simultaneously in all the centromeres of the cell. For this reason it is possible that this effect need not be reflected as an intensification of the pumping activity in the whole spindle. In any case it should be worth while to look for especially rapid movements of small granules lying close to the traction fibers of centromeres that are in this special stage. Thus far such studies have not been made.

With the beginning of telophase it may be seen in plant cells that acentric bodies again start to make regular poleward movements within the spindle that is now called the phragmoplast (FIGURE 5). This effect has been directly observed in endosperm cells studied in the living state (Bajer and Molè-Bajer, 1956; Bajer, 1958b; Molè-Bajer and Östergren, in preparation) and it may be concluded from observations of fixed material (irradiated material of *Allium cepa*, *Hyacinthus orientalis*, and *Tradescantia pedicellata* studied by Östergren,

unpublished). This effect can also be seen to act on lagging univalents in hybrids (for example, in *Narcissus*, Östergren, 1957). It also acts on chromosome arms hanging down in the phragmoplast from the polar groups, with the result that these arms are pushed in the polar directions. This effect on the chromosome arms was noted by Heitz (1943) and interpreted by him as evidence for a *Stemmkörperwirkung*. There is, however, no spindle stretching at this stage so the effect cannot be due to a *Stemmkörperwirkung* in the ordinary sense (Bajer, 1958b).

The acentric bodies inside the phragmoplast behave in very much the same way here as they do in the half spindles of prometaphase, metaphase, and anaphase, and in our opinion they are moved here, too, by the same kind of pumping activity. However, as there are no centromeres in the equator of the phragmoplast, it might be thought that the pumping activity in this case could not be provoked by an influence from traction fibers on the spindle material.

Nevertheless it is in our opinion a very probable interpretation that the phragmoplast, too, contains traction fibers similar to the chromosomal fibers. Our interpretation is as follows: it may be seen (for example, in phase contrast pictures from endosperm cells of *Haemanthus*) that the formation of the new cell wall begins with the appearance of small granules in the equator of the phragmoplast. These granules are attached to fibers in a way that looks very similar to the attachment of centromeres to chromosomal spindle fibers. The granules later form the new cell wall in one way or another (either they fuse to give rise to it or they produce it through some kind of secretory activity). In any case, when these granules are to produce the new wall it is necessary for the cell to coordinate their positions very strictly, so that they are all placed in one level. It is a reasonable hypothesis to suppose that the cell performs this coordination by means of having the granules attached to traction fibers that work in the same way as in the chromosomal mitosis and put the granules in a kind of metaphase plate. This would explain why acentric bodies located in the phragmoplast move out of it in the same way that they do in the mitotic half spindles. The interpretation assumes that there are similar traction fibers in both of these structures and that these traction fibers provoke a pumping activity of the same kind. This hypothesis is also in agreement with the strong double refraction observed by Inoué and Bajer (manuscript in preparation) in the phragmoplast that in this way resembles the chromosomal spindle fibers of metaphase and anaphase.

This interpretation is independent of what we may assume concerning the origin and the fate of these cell wall-forming granules in the phragmoplast. In whatever way they originate the cell has to face the problem of putting them in the proper position so that they can form a continuous wall, and the above hypothesis may represent the way in which this problem was solved.

The effect of this pumping activity working in the mitotic spindle is modified by a factor of another kind which we might call the elimination forces. These can be seen both in plant and animal cells to act on all sorts of bodies in the spindle, striving to move these out of the organized spindle region. Concerning the mechanism for the origin of the elimination forces it is possible only to make suppositions, but their existence is clear from the behavior of chromosomes and other bodies involved. The forces are presumably caused by a dis-

turbance provoked by the presence of foreign bodies in the dynamic equilibrium of the spindle system, which strives to restore its equilibrium by producing forces acting to remove the disturbing bodies, an interpretation suggested by Östergren (1945*b*). Surface tension at the surface between the spindle and the cytoplasm may or may not contribute to the effect.

Before discussing the effects of the elimination forces may we point to the difference found in the arrangement at metaphase in plant cells of long thin chromosomes on one side and of short contracted chromosomes on the other (FIGURES 6*a* and *b*). The centromeres and the most proximal regions of the arms are in both cases maintained more or less effectively in the plane of the metaphase plate, an effect that no doubt is due to the pulling forces by means

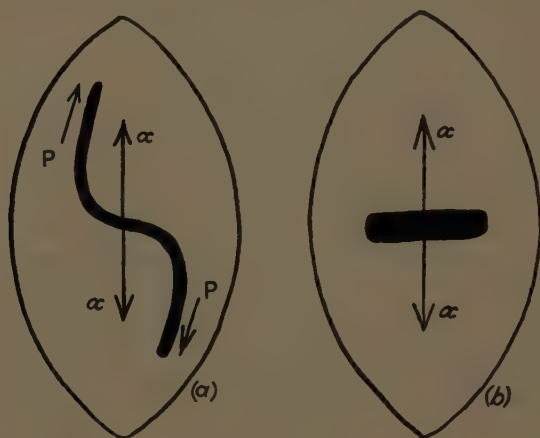


FIGURE 6. The difference in arrangement at metaphase in plant mitoses between long thin chromosomes (*a*) and more contracted ones (*b*). The poleward pull from the traction fibers is denoted by α . When the chromosomes are thin and easily flexible the pumping activity (*P* in the figure) bends the arms in the poleward directions, but when the chromosomes are more contracted and stiff they remain in a more equatorial arrangement along their whole length. Such differences in chromosome arrangement as between the figures (*a*) and (*b*) can in many plants (for example, the hyacinth) be found between root tip mitoses and pollen grain mitoses.

of which the chromosomal spindle fibers act on the centromeres in the two opposite poleward directions. These pulling forces were called the α -factor by Östergren (1949*c*) for which reason they have been marked with the letter α in FIGURES 6*a* and *b*. In the case of long thin chromosomes, however, the arms are bent rather close to the centromeres and the main part of each arm is straight and directed toward the nearest spindle pole, an effect that, as already mentioned, no doubt should be due to the same mechanism that also causes the movement towards the poles in prometaphase and early metaphase of acentric fragments, pieces of nucleoli and small granules, that is, it is due to what has been called in this paper the pumping activity (marked *p* in FIGURE 6*a*). A chromosome that is more contracted and thus shorter and thicker is, on the other side, usually arranged with its whole body lying more or less flat in the equatorial plane (FIGURE 6*b*).

A very reasonable interpretation of the difference in the arrangement described by FIGURES 6*a* and *b* is that it is due simply to the fact that the thin chromosomes are much more flexible and that the poleward forces acting on the arms are then capable of bending them into the arrangement of FIGURE 6*a*, while the more contracted chromosomes in FIGURE 6*b*, because of their stiffness, are much more difficult to bend in this way. This interpretation actually has already been suggested by Östergren (1949*a*) who, however, failed to realize the existence of the pumping activity. Differences as between FIGURES 6*a* and *b* are found in many species of higher plants (such as in *Hyacinthus* and in *Allium*) when root-tip mitoses are compared with pollen-grain mitoses.

When the chromosomes have the equatorial arrangement of FIGURE 6*b*, they are probably located with their whole lengths in the "dead zone" in the equator (compare FIGURE 2*b*), a region without pumping activity. This opinion, of course, is in no way in conflict with the opinion that their equatorial arrangement is the consequence of their stiffness that has made them straight combined with the poleward pull at the two chromatid centromeres that gives the proximal regions of the chromosomes an orientation within the equatorial plane. These two factors (stiffness and centromere pull) have overcome the pumping action that should have worked in the poleward direction on various regions of these chromosomes before they became located in the equator along their whole length.

Furthermore, it should be mentioned that there are, of course, also various intermediate types of arrangement that can be found between the two diagrams of FIGURES 6*a* and *b*, arrangements corresponding to intermediate degrees of chromosome contraction. Such intermediate types are actually the most commonly occurring among the root tip mitoses of higher plants.

When the chromosomes are lying flat in the plane of the equator at metaphase they show the kind of arrangement illustrated by the diagrams of FIGURES 7*a* and *b*. At least the chromosomes that lie on the periphery of the metaphase plate are bent at their centromeres and in the proximal regions of their arms, and they lie oriented and with their centromeres inward toward the center of the metaphase plate with their arms outward toward the cytoplasm and often, actually, in the cytoplasm. Their bent shape that remains as a characteristic feature in chromosomes that have otherwise lost their prophase bends (from relic coils and irregular arrangement inside the nuclei) suggests that these chromosomes are influenced by antagonistic forces acting in the transverse direction of the spindle (Östergren, 1945*b*), that is, the centromeres are influenced by forces pulling them in the centripetal direction inward toward the center of the metaphase plate while, on the other hand, the chromosome arms are subjected to forces acting on them in the centrifugal direction away from the center of the metaphase plate.

It is easy to realize that the poleward pull acting on the centromeres (α in FIGURES 7*a* and *b*) should have a transverse component, called α (T) in the diagrams, that is directed inward toward the axis of the spindle. The outward bending of the chromosome arms suggests that this inward force is balanced by an outward force striving to eliminate the chromosome arms from the spindle (Östergren, 1945*b*). The same situation is valid for bivalents at meiosis as illustrated by the diagram of FIGURE 7*c*, and reported for meiosis in rye

by Östergren and Prakken (1946); it can, of course, be seen very well also in numerous other materials. A similar situation is even found in univalents at meiosis, which are found to be bent in a corresponding way, even though they are very little constricted in their centromere regions and although they are

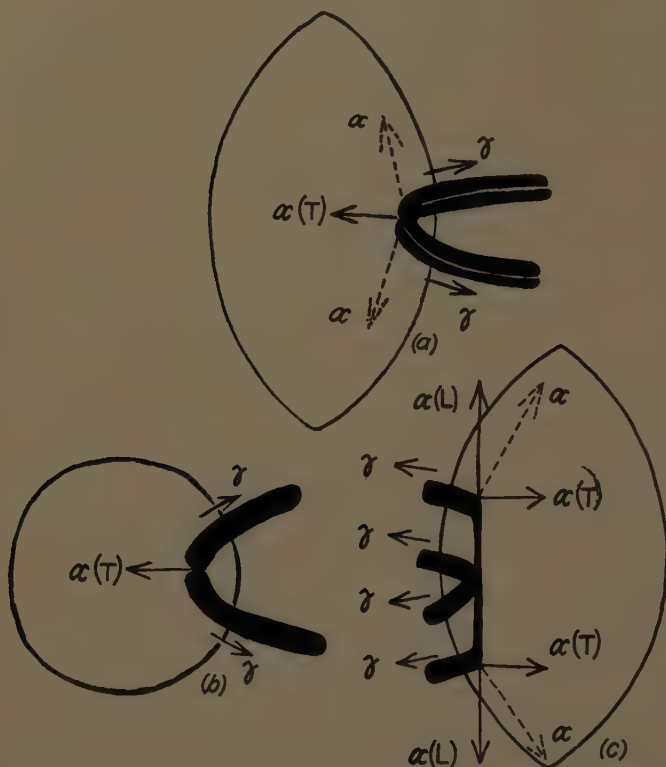


FIGURE 7. Diagrams of certain aspects of the chromosome arrangement at metaphase in higher plants. (a) Mitosis, side view. (b) Mitosis, polar view. (c) First meiotic division, side view. (a-c) The poleward pulling forces of the traction fibers have been denoted by α . These forces have longitudinal components $\alpha(L)$ and transverse components $\alpha(T)$. The chromosome arms are influenced by the elimination forces (called γ in the figures following the terminology of Östergren, 1949c). These forces strive to remove the arms out of the organized spindle region. The transverse component of the poleward pull together with the elimination forces that are acting in the opposite direction are the main factors in causing the characteristic bent shape of chromosomes lying on the border of the metaphase plate and in causing the outward bending of free arms of meiotic bivalents. The effects shown in a and b are especially well expressed in cells where the chromosomes are lying more or less flat in the equator (an arrangement corresponding to that in FIGURE 6b).

strongly contracted; for this reason they should be especially difficult to bend (Östergren, 1951).

There are some data which suggest that in animal cells, where there are centrosomes and more or less well-developed asters, there are strong forces acting on the chromosome arms striving to move them away from the centrosomes. These forces acting to move bodies away from the centrosomes are probably

of the same kind, or of a closely related kind, as the elimination forces striving to move the chromosome arms out of the spindle in plant mitoses.

Thus the chromosome behavior that may be seen in the commercially available cinematographic film by Bloom and Zirkle on mitosis in the newt (cited in the list of references, below) demonstrates the existence of such forces acting on the chromosome arms in the direction away from the centrosomes. The second cell in their film shows very clearly how the chromosome arms are pushed away by the asters in early prometaphase, an effect that produces an empty space around each of the centrosomes. A chromosome arm that has by chance come to lie on the other side of the centrosome (away from the equator) cannot be pulled through the astral region when the centromere of the chromosome moves towards the equator, but must be dragged around the aster.

In the Bloom and Zirkle film on mitosis in the newt there are some chromosomes (variable in number from one mitosis to another) that show a delayed congression in the metaphase plate. These chromosomes, that were called centrophilic chromosomes (Bloom *et al.* 1955; Zirkle, 1959) show a pronounced attraction of their centromeres to one or the other of the centrosomes (see the diagram in FIGURE 8*a*). Whatever the explanation* of this unipolar attraction may be, the behavior of the arms of these chromosomes is in good agreement with the idea that the chromosome arms are repelled from the centrosomes in this material.

These centrophilic chromosomes are strongly bent with their centromeres directed inward toward the centrosomes and their arms directed outward, and they retain this shape and arrangement when changing their position between different sides of the centrosome. This shape in some cases could be due in part to an active movement of the centromeres toward the centrosomes, but this is scarcely the only factor producing this effect, as these chromosomes often move but slightly and still develop and retain this shape. Most probably the shape of these chromosomes is due largely to a balance between a force working in the outward direction on the arms and another force working in the inward direction on the centromeres toward the centrioles (the traction-fiber pull).

Although the chromosomes in the newt mitoses are very long and thin they have a quite different arrangement in the metaphase plate than is found in the case of long thin chromosomes in plant mitoses. In plant cells the arms of long thin chromosomes are lying within the spindle and are "pumped" in the poleward direction as illustrated in FIGURE 1*d*, but in the newt mitoses the chromosomes show a strong tendency toward being located only on the periphery of the metaphase plate (the well-known "hollow spindle" type of mitosis

* It appears very probable that the metaphase chromosomes of mitosis in the newt have chromatid centromeres that are capable of some degree of independent individual orientation. In that case such a chromosome would become arranged for normal separation through a process of co-orientation of chromatid centromeres rather than through an auto-orientation of a chromosome centromere, and the centrophilic chromosomes would be in a situation similar to that of certain meiotic bivalents in which the centromeres of both partners are oriented toward the same pole during prometaphase (Östergren, 1951). It is clear that a process of co-orientation similar to that found at meiosis could occur also at mitosis. Östergren's theory of co-orientation was actually already applied to mitotic chromosomes in *Ulophyesma* by Melander (1950). In this connection we meet some problems of the orientation stability of different arrangements which, however, cannot be discussed here.

known from numerous illustrations from newts and salamanders) and these chromosomes are strongly bent close to the centromeres and have the arms protruding radially outward from the metaphase plate into the cytoplasm. This difference in the arrangement could be due to a strong action of the elimination forces in the newt type of mitosis and to their working not merely out of the spindle but also away from the centrosomes.

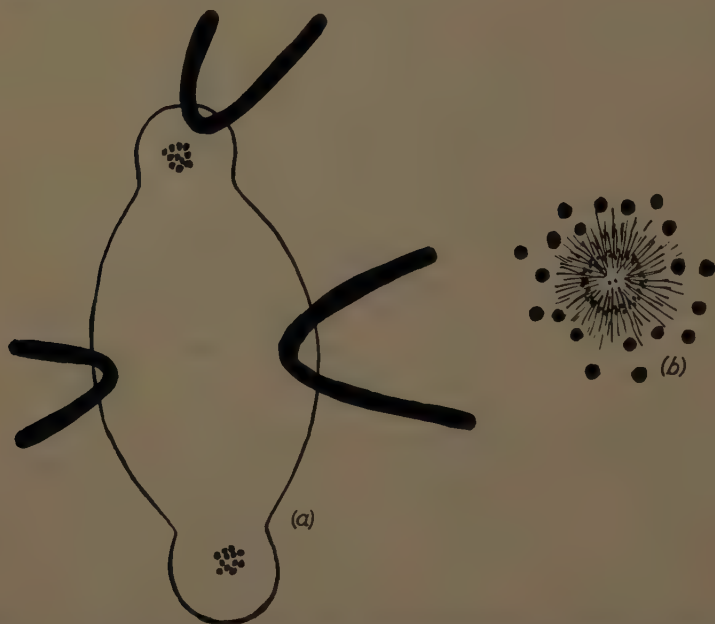


FIGURE 8. Diagrams of some effects of the elimination forces and of the pumping activity in animal cells. (a) In the film studies by Bloom and Zirkle on mitosis in the newt it was found that certain chromosomes (called centrophilic) remain in the asters for a long time before they go to the equator. These chromosomes show evidence from their shape of a pulling force acting on their centromeres toward the centrosome of the aster while the chromosome arms appear to be influenced by the elimination forces working in the opposite direction. In one cell (Bloom *et al.*, 1955) it was found that many small fat droplets accumulated at the centrosomes, an effect that may be due to what we call the pumping activity. (b) In various animal cells depicted by Wilson (1925, it is shown how small granules may enter the asters where they are stratified in spherical layers; on the other hand, yolk bodies (which are larger) are kept outside the asters. This effect could result from the combined action of the elimination forces and the pumping activity, if the elimination effect increases strongly with an increasing size of the bodies.

The pumping mechanism working in the poleward direction could be the same in these plant and animal mitoses, and in both cases it could produce the poleward pull acting on the centromeres through the intermediation of the chromosomal spindle fibers. This pumping mechanism should in both cases also act in the poleward direction on the chromosome arms, but in the animal cells there are strong "elimination forces" acting on the chromosome arms in the direction away from the centrosomes, and these elimination forces counteract and overcome the pumping activity to such an extent that the influence of

this activity cannot be detected on the chromosome arms in the newt mitoses (and in many other animal mitoses).

It is of interest in the present connection that in one cell of the newt Bloom *et al.* (1955) found numerous small granules or fat droplets that were attracted to the centrosomes and accumulated around them. This type of behavior can be studied in the last cell in the film of Bloom and Zirkle. This movement of small granules toward the centrosomes in this newt cell might be interpreted as due to the same pumping activity working in the poleward direction that causes the movement of chromosome arms and acentric bodies in plant cells in that direction. Now, however, the chromosome arms in the newt cells are being repelled from the centrosomes. This difference in the behavior between the granules and the arms might, in our opinion, be due to the difference in size of these different bodies.

If the pumping activity works in about the same way on small and large bodies while the elimination forces grow much stronger with an increased size of the body, we could very well get the effect of small bodies moving toward the centrosomes while large bodies are repelled from them. The traction fibers of the centromeres that are very thin should probably cause only very weak elimination forces (if any) while, on the other hand, the large surface of these fibers should catch much of the pumping activity and cause them to produce a vigorous pull on the centromeres.

In this connection it might also be of interest to point to some observations on various animal materials cited by Wilson (1925) showing how yolk bodies (that are large) are kept out of the astral regions while various small granules penetrate into the asters and are deposited inside them in spherical zones. This situation has been schematically indicated in our diagram (FIGURE 8b).

The occurrence of elimination forces that act in the direction away from the poles in animal materials as opposed to the absence or much weaker development of such forces acting from the spindle poles in higher plants may be due to the presence in the animals of centrosomes and the absence of such bodies in plants. It is possible, however, that the effect may not result from the centrosomes as such, but rather from the relatively large and well developed asters that are often found in materials having centrosomes. This question might be studied by making observations on the chromosome behavior in the early endosperm mitoses of *Crepis capillaris*, where there are distinct asters (without centrioles) already in metaphase (Östergren, 1954). In most species of higher plants the asters (or corresponding structures) develop to a noticeable degree only in the latest stages of mitosis (Östergren *et al.*, 1953; Östergren 1954); for this reason we should not expect them to influence the chromosome arrangement at metaphase. Observations of this kind on the chromosome arrangement in the endosperm mitoses of *Crepis* thus far have not been made.

In the case of mitoses having elimination forces acting away from the centrosomes or spindle poles we should not expect persisting nucleoli and similar bodies to move in the poleward direction within the spindle, but they should be eliminated laterally from the spindle. There are also found many figures in the literature from a big variety of different organisms showing a lateral arrangement of such bodies relative to the mitotic figure, for example, in Bělař (1926;

1928); Tischler (1921–1922). It is, however, possible that the figures found in these works often may not show the result of an actual lateral “elimination,” but rather the situation resulting from the development of a central spindle beside the nucleus (or beside the nuclear content) after which the chromosomes become attached to this spindle, while, of course, the nucleolus remains unattached lying at the side of the mitotic figure. Even in that case, however, the position of these bodies more or less equatorially at the side of the spindle is in good compatibility with the action of these elimination forces that are working in the directions out of the spindle and away from the poles.

The Feulgen-positive masses (*Nukleinkörper*) that were found by Bayreuther (1956) to appear during the oögonial mitoses in various Tipulids also occupy a position in the peripheral region of the spindle equator during metaphase. During anaphase they at first retain their peripheral position and lag between the separating chromosome groups; finally, in late anaphase, they move in the poleward direction and join one of the telophase nuclei. The metaphase and early anaphase positions of these nuclein bodies are in our opinion likely to be due to the elimination forces already discussed; it should be noted that these bodies are comparatively large in size. The late anaphase behavior of these bodies looks very similar to the movements of acentric fragments in the phragmoplast of plant cells. It is not unlikely that the influence of the centrosomes in causing elimination forces directed toward the equator will decrease or disappear in the interzonal spindle region at late anaphase and telophase, as this spindle region then may not connect to the centrosomes but may end at the two daughter nuclei. Because of this change, bodies inside or in contact with the interzonal region could then be permitted to move in the poleward direction.

Also in animal cells there is often found an arrangement of small granules in the equator of the interzonal spindle region at telophase, a situation that resembles that of the cell wall-forming granules in plant phragmoplasts. The appearance of such equatorial granules therefore need not necessarily imply the formation of a new cell wall. Possibly the appearance of such granules could have something to do also in these animal cells with the origin of a pumping activity in the interzonal spindle region acting to move bodies in the poleward direction. It is possible, however, that the pumping activity in some animal cells at the late anaphase to early telophase stage might rather have another origin, since it is often found that this activity has a distinctly polarized character. This is found in the above-mentioned oögonial mitoses of the Tipulids from which Bayreuther (1956) reports that the nuclein bodies move in the same direction in neighboring cells. It is also found during meiosis on the male side in Tipulids, where it is found that the unpaired and lagging X and Y chromosomes regularly separate toward opposite poles in late anaphase (Dietz, 1956). A similar mechanism also causes a directed nondisjunction of X-chromosome trivalents at male meiosis of some Ostracods (Dietz, 1958). We have no idea concerning what may cause the polarized character of these movements, but their general resemblance to the movements of acentric fragments in plant phragmoplasts makes it necessary to mention these effects in the present connection.

The observations of Stich (1954) on the mitotic behavior of Feulgen-positive granules that are eliminated during the early embryonal mitoses in *Cyclops*

can be interpreted in accordance with the ideas of this paper. In prometaphase, when these granules are considerably smaller than the chromosomes (Stich, 1954), they are found to move toward the spindle poles at the same time that the chromosomes move toward the plate. These granules accumulate in the polar regions (probably without coming very close to the centrosomes, if we can judge from the drawings). Here they occupy a peripheral position close to the surface of the spindle that, according to Stich, is still enveloped by the nuclear membrane. This behavior of the granules is in agreement with our opinion that the elimination forces acting away from the centrosomes are weaker on smaller bodies; for this reason such small bodies can be transported in the poleward direction by the pumping activity in the metaphase spindle. The position of such small bodies should result from an equilibrium between the pumping activity acting toward the poles and the elimination forces acting to move the bodies away from the poles.

During the progression of mitosis these granules were found by Stich to increase in size through fusion with one another and through swelling; in late metaphase and during anaphase the granules were found to move toward the equatorial plate. If the elimination forces are very dependent on the size of the bodies, as we suppose, then this behavior might be expected, and it should be due to a shifting of the equilibrium of the particles caused by the increased action of the elimination forces resulting from the larger size of the particles. As the pumping activity disappears in the interzonal region (FIGURE 2c) the movement toward the plate from the elimination forces should be even more pronounced in anaphase than in late metaphase. During anaphase the granules were found to accumulate in the peripheral region of the equator where they continued to increase in size by fusion.

During late anaphase and telophase in the *Cyclops* mitoses the spindle grows much narrower; the equatorially arranged granules are taken up in the central part of the spindle, stretch in the longitudinal direction, and move in the two opposite poleward directions: a movement that causes them finally to accumulate close to the telophase nuclei. The late-anaphase and telophase behavior of these granules is closely similar to that of acentric fragments in the phragmoplast of higher plants. The stretched granules inside the interzonal spindle region often take on a constricted dumbbell-like shape similar to that sometimes found in the case of persisting nucleoli at metaphase in higher plants (FIGURE 1c). The movements of the *Cyclops* granules at telophase and also of moving acentric bodies in plant mitoses are probably due to the same kind of pumping activity. The reason why the granules are taken up in the central part of the spindle at this time is a riddle, however. This effect may be accounted for by making additional assumptions but as the value of these suppositions cannot be judged at present it would not be very useful to discuss them.

Stich (1954) explained these movements of granules during mitosis as resulting from circulating currents within the spindle. Now, however, the granules are not merely floating around, but they are found in repeated instances to accumulate in special positions: at first, close to the poles; later, in the equator; and, finally, close to the telophase nuclei. This behavior is in our opinion more compatible with our interpretations than with the hypothesis that they are carried by simple currents.

According to the present interpretation the same basic factors could be involved, to a large extent, in the mitotic processes both of various animals and of higher plants, and the differences would mainly consist in a variation in the relative importance of the different factors from one material to another combined with some not too important modifications in the structural organization of the spindles due to the presence of centrioles in certain types of mitosis and their absence in others.

The present interpretation assumes that the movement of acentric bodies inside the spindle results essentially from the production by the spindle substance of a shifting or sliding force acting between the included body and the spindle, and the same mechanism is supposed to move also the centromeres; in this case the main action of the sliding force is to move the chromosomal spindle fiber relative to the background material of the spindle. We call the mechanism causing this effect the pumping activity of the spindle.

Although this idea does not assume that the mitotic movements are due to cytoplasmic currents, it should be noticed that the kind of mechanism assumed by us for mitosis could very well be the same as that causing cytoplasmic streamings. Kamiya (1959) is of the opinion that the cytoplasmic streamings result from the generation of a sliding force at the border between the cortical gel and the liquid endoplasm. He also believes that the same kind of mechanism acts to cause the movements of various bodies that perform so-called independent movements within the endoplasm. These movements, too, could result from the generation of a sliding force at the surface between the gel structure of the bodies and the endoplasm.

It is very well possible that the mechanism causing the sliding force in cytoplasmic streaming and in autonomous movements of bodies inside the cytoplasm could be the same as our pumping activity within the spindle of mitosis. This may be an elementary mechanism that the cell has at its disposal and which can be applied to perform many different functions. Another phenomenon that may be related is expressed in the creeping movements of cells (for example, diatoms) on their substratum and, according to the so-called sliding model of muscle contraction formulated by J. Hanson and H. E. Huxley and A. F. Huxley (Weber, 1958), a similar mechanism may work also in muscles.

It was realized by Kamiya (1959) that the shifting mechanism acting during cytoplasmic streaming could be a more general mechanism for different protoplasmic motions and he also mentioned, among other motion phenomena, the behavior of centromeres in nuclear division.

Summary

The centromeres are not the only bodies that move in the spindle at mitosis. In plant mitosis it is found that acentric bodies of various kinds also perform characteristic movements. During prometaphase these bodies regularly move toward the poles and accumulate in the polar spindle regions or pass into the cytoplasm. These movements are not due to cytoplasmic currents within the spindle, since the acentric bodies can be accumulated at a pole, and a current would not be able to dispose them in one place, but as it continues to flow it would carry the bodies along with it.

We think that the acentric bodies are transported by a kind of pumping activity by means of which the spindle substance transports materials within it, while remaining stationary itself. During metaphase the pumping activity works from the equator in the two opposite poleward directions. We suppose that this pumping activity is the factor that produces the poleward pull working on the centromeres and that it does so by pumping at the chromosomal spindle fibers.

The chromosomes are propelled in very much the same way as sailboats. The pumping activity is the wind and the chromosomal spindle fiber is the sail. The centromeres and their traction fibers differ from the acentric bodies, however, in so far as they are not merely carried passively by the pumping activity. There is evidence for the opinion that they actually provoke this activity and determine its direction.

During telophase there is a similar pumping activity at work in the phragmoplast, where it can be seen to carry acentric bodies in the poleward directions. This is probably due to a presence of traction fibers in the phragmoplast that are here attached to the small granules that are to form the new cell wall. The traction mechanism has here the purpose of arranging these granules in a single flat level in order to make it possible for them to form a continuous cell plate.

The effect of this pumping activity is modified by a factor of another kind that we might call the elimination forces. These may be seen in both plant and animal cells to act on all sorts of bodies in the spindle, striving to move these out of the organized spindle region. In animal cells these forces are often found to work strongly in the direction away from the centrosomes. In this way the elimination forces may counteract and conceal the effect of the pumping activity on acentric bodies to such an extent that this activity cannot be detected. In spite of this it is reasonable to suppose that the mechanisms of the transport phenomena found at mitosis are essentially the same in plant and animal cells.

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NUCLEIC ACID SYNTHESIS IN RELATION TO THE CELL DIVISION CYCLE*

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Introduction

The cyclic nature of DNA (deoxyribonucleic acid) synthesis in relation to cell division is now well established. The evidence that synthesis regularly occurs in interphase and coincides with the duplication of the chromosomes has been reviewed previously (Taylor, 1957). Studies on the distribution of tritium-thymidine incorporated into chromosomes show that duplication is accomplished by separation of the two original DNA subunits of each chromosome and by the synthesis of two new subunits. At the following division each chromatid (daughter chromosome) receives one original and one new subunit. The new subunit labeled by the tritium in each chromosome is passed on intact to one of the daughter chromosomes descended from these labeled chromosomes (Taylor *et al.*, 1957). The subunits are conserved at each duplication and only become disrupted by an occasional sister chromatid exchange, that is, exchange between the daughter chromosomes that probably occurs during duplication (Taylor, 1959a).

Cyclic changes of RNA synthesis in relation to cell division are less well understood and appear to be less predictable. A few instances in which RNA synthesis stopped or fell to a low level during chromosome duplication have been reported (Taylor, 1958, 1959b; Siskin, 1959). However, this is not a regular occurrence (Woods and Taylor, 1959) and there are many types of cells in which the synthesis of both types of nucleic acids proceeds simultaneously.

One cyclic change that appears to be regular is the failure of cells with condensed chromosomes to form new RNA. In several types of cells indications that RNA synthesis stops during the middle stages of division have been observed (Taylor, 1958 and 1959b).

By the use of autoradiography and tritium-labeled nucleosides of high specific activity, especially with cells in sterile culture, a more critical analysis of the cyclic changes in RNA synthesis is now possible. The objective in the present work was to obtain additional information on the site of synthesis of RNA and to study cyclic variations in relation to the division stages.

Materials and Methods

Two strains of connective tissue cells (♀ 1404 and ♂ A1290) isolated from embryonic tissues of Chinese hamsters by George Yerganian, Children's Cancer Research Foundation, Boston, Mass., have been used in these studies. The cells have maintained a near diploid chromosome number ($2n + 1$ or 2)

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during several months in culture. During these experiments they were grown in Eagle's medium with 15 per cent calf serum. Tritium-labeled thymidine (sp. act. 1.8 curies/mm.) and cytidine (sp. act. 360 mc./mm.) were mixed with a medium in which cells had been growing for 18 to 24 hours. All cultures were grown attached to cover glasses in 5 ml. of medium in small Petri dishes. These were maintained at 37° C. in an atmosphere of 5 per cent CO₂ in air. In most of these experiments the cover glasses with cells attached were immersed in the labeled medium (0.1 μ c./ml. for thymidine-H³ and 1.0 μ c./ml. for cytidine-H³) for 5 to 10 min. and, after rinsing in a medium with excess unlabeled nucleosides, were allowed to grow in the presence of an excess of the unlabeled nucleosides to quickly dilute any remaining labeled precursors. Thymidine at 100 times the concentration of the thymidine-H³ was added to a medium in which cells had been growing for 18 to 24 hours. To dilute the cytidine-H³ and its derivatives, unlabeled cytidine and uridine, each at 25 times the molar concentration of the cytidine-H³, were added to a similar medium.

Cells were fixed for 5 to 10 min. after rinsing a few seconds in a balanced salt solution. Fixatives used were alcohol-acetic acid (3:1) or an alcoholic Bouin's fluid (80 per cent ethanol, 150 cc.; formalin, 60 cc.; glacial acetic acid, 15 cc; picric acid, 1 gm.). The cells were either air-dried or transferred to 95 per cent ethanol after fixing. After appropriate extraction of the cells with cold TCA (trichloroacetic acid) to retain RNA, and 1 N HCl at 60° for 7 min. or after digestion in ribonuclease for the removal of RNA (Taylor, 1959b), the preparations were again rinsed in 95 per cent alcohol and air-dried. The cover glasses were fixed by the use of Euparal or Diaphane to slides with the cells exposed. When the preparations had dried for a few days the slides were coated with Kodak AR-10 stripping film. The autoradiograms were developed after appropriate exposure (3 days to 3 weeks). HCl-hydrolyzed material was stained by the Feulgen reaction before applying the film. The slides with unstained cells were developed and stained according to the following schedule with all solutions at 18 to 20° C.: 5 min. in one-half strength D-19 at 18° C.; 30 sec. in water for rinsing off developer; 10 min. in one-half strength Kodak acid fixer; 5 min. in water for rinsing off excess hypo; 2 min. in Kodak hypo clearing agent (one-half strength); 5 min. in several changes of fresh water; 20 min. in a staining solution (20 mg. of azure B bromide and 0.5 gm. of potassium acid phthalate in 100 ml. of distilled water); rinse for 20 to 30 sec. in water and air dry.

The stripping film was applied by floating pieces of the appropriate size on water at 25 to 26° C. The film was also dried at this same temperature. The control of temperature is important, for the film should remain expanded during drying to approximately the same extent that it will expand in the developing solution. Otherwise the film may become loose and fall off the slides.

Results

The duplication of the chromosomes (synthesis of DNA) in relation to the cell division cycle. Details of this experiment have been described elsewhere (Taylor, 1960), but a summary of the findings are necessary as a basis for the studies

on RNA synthesis. Cells were exposed to thymidine- H^3 for 10 min., rinsed and transferred to a medium with excess unlabeled thymidine. Samples were fixed at intervals and examined with respect to the percentage of labeled interphase nuclei and division figures. Other slides were handled in a similar way except that the contact with thymidine- H^3 was 30 min. These slides were used to show that the labeled precursors were depleted or diluted within less than a minute. Grain counting in the autoradiograms showed that no detectable amount of label DNA accumulated after removing the cells to the medium with excess unlabeled thymidine. The H^3 already incorporated into DNA was, however, all retained and equally distributed to each two daughter cells at division.

By counting the labeled and unlabeled interphase nuclei and division figures, the data shown in FIGURE 1 were collected. After a 10-min. labeling period, 43 per cent of the interphase nuclei were labeled. Labeled division figures did not appear for nearly two hours. This measures the shortest interval between the end of DNA replication and late prophase or metaphase. Within three hours nearly 100 per cent of the cells reaching division contained labeled chromosomes. This indicates that the interval between the end of synthesis and division is usually between two and three hours. The population of labeled cells continued to divide during the next 6 or 7 hours. However, by the eleventh hour after labeling nearly all labeled cells had divided. Most of the cells dividing between the eleventh and twelfth hours were at stages preceding DNA synthesis during the contact with thymidine- H^3 . The few labeled division figures were made up of cells that were slow in dividing and a few that were already at the second division after labeling; these had only one chromatid of each chromosome labeled. By twenty hours the frequency of labeled division figures was again at a peak (FIGURE 1). The length of one cycle can be estimated from the interval between the two peaks and is approximately 14 hours. The average time the cell spends in DNA replication can be calculated from the percentage of cells that were incorporating thymidine- H^3 at any instant in the cycle. When the percentage of cells labeled after 10 min. is multiplied by the average generation time (0.43×14 hrs.) an interval of approximately 6 hours is obtained for the period of DNA replication. The division stages from mid-prophase to mid-telophase occupy about 30 min. (see section below). If 6 hours is taken as the average replication time, $2\frac{1}{2}$ hours as the interval between replication and late prophase and one-half hour for the remainder of the division stages, the difference between the sum of these times and 14 hours is 5 hours, the postdivision interval before another replication begins.

The shaded portion of the bars in FIGURE 1 shows the percentage of division figures with more than one half of the length of the chromosome complement labeled by the thymidine- H^3 . Duplication of the chromosomes in the cells of Chinese hamster is asynchronous for various chromosomes and different sectors of individual chromosomes. Contact with thymidine- H^3 for only 10 min. of a duplication cycle of 6 hours reveals the pattern of the asynchronous duplication. Details have been reported previously (Taylor, 1960). The Y chromosome, the long arm of the X chromosome, the short arms of 2 medium-

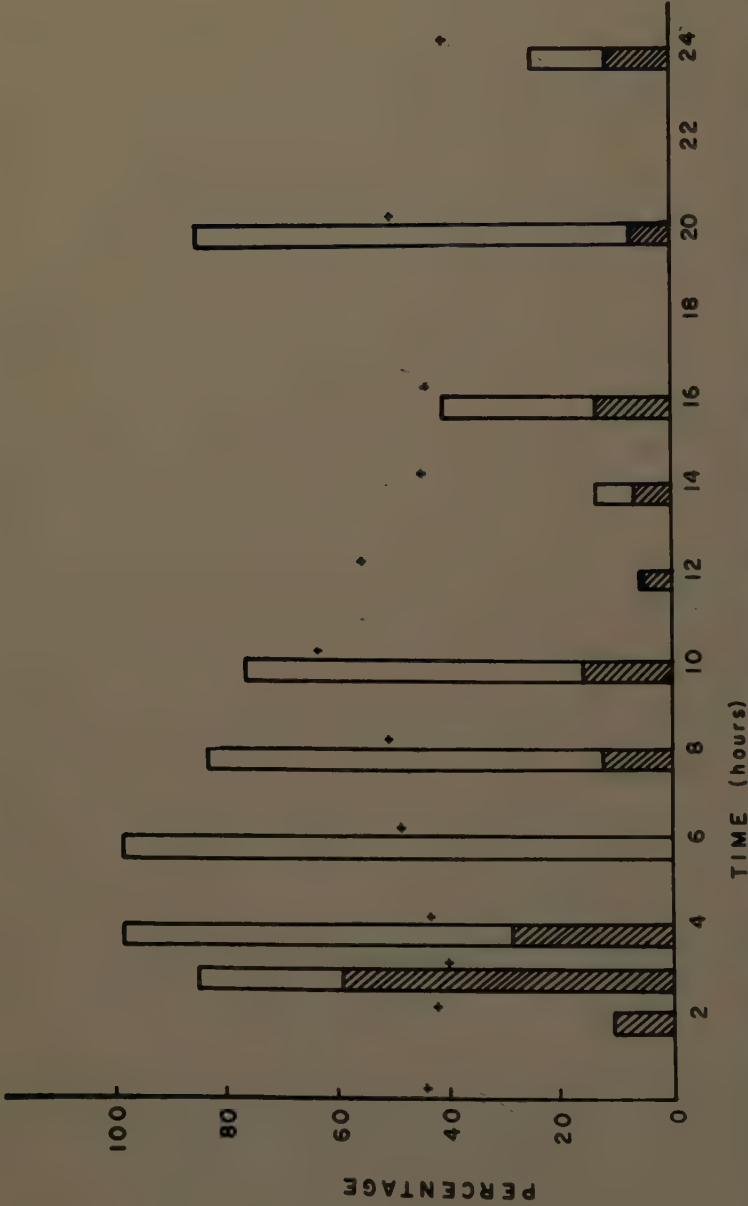


FIGURE 1. Graph showing the percentage of labeled interphase nuclei (*crosses*) and of labeled division figures (*bars*) in cultures of Chinese hamster cells at various intervals after a 10-min. contact with a medium containing thymidine- H^3 . Divisions were accumulated for one hour before fixing by addition of colchicine. The shaded portion of the bars shows the percentage of division figures in which less than one half of the total chromosomal complement was labeled (Taylor, article in press).

sized chromosomes, and the major portions of 2 pairs of small chromosomes were regularly duplicated in the last half of the period of DNA synthesis. When the thymidine- H^3 was available only during the last 30 min. of the period of DNA replication nearly all of the isotope incorporated was in these chromosomes.

The sequence of duplication was similar in the two strains of cells examined, except that the 2 X chromosomes of the female strain were different. One had the pattern typical of the X chromosome in the male, that is, the short arm duplicated early and the long arm late. However, the other X chromosome had both arms duplicated late.

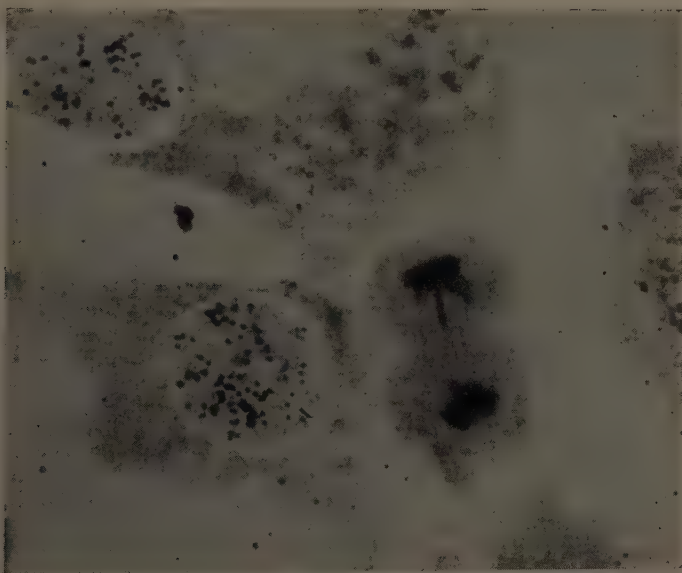


FIGURE 2. An autoradiogram showing cells fixed immediately after a 10-min. period in contact with a medium containing cytidine- H^3 . Note that only interphase nuclei are labeled; cells in division do not incorporate the cytidine into RNA.

The synthesis of RNA during the cell cycle. When the cytidine- H^3 was supplied to the Chinese hamster cells for 5 to 10 min., only the nuclear RNA was labeled (FIGURE 2). The slides from which data were collected were exposed to the film for 10 days and there was an average of 86 grains over the nucleus (TABLE 1), and none detectable above background over the cytoplasm. Even when the cells were exposed to the film for 21 days, the label in the cytoplasm was not detectable. Therefore, a real lag period occurs before any labeled RNA appears in the cytoplasm. This should not occur if the difference in labeling of RNA in cytoplasm and nucleus were due to differences in turnover rates. Both should be labeled simultaneously and the amount of label in both should increase linearly until equilibration begins to produce a differential effect. The lag indicates that synthesis of RNA occurs only in the nucleus and is later transferred to the cytoplasm. This hypothesis is supported by the fact that essen-

tially all of the label RNA has disappeared from the nuclei within four hours (FIGURE 3). Only cells that were in the period of DNA replication (about 43 per cent) take up tritium that is retained in the nucleus. Most if not all of this is incorporated into DNA.

However, to get additional evidence concerning the transfer hypothesis, the changes in distribution of the labeled RNA was observed at intervals after the removal of cells to a medium without cytidine- H^3 . A group of cultures were prepared from a population of cells growing in 1 culture vessel. After the new cultures had been growing for about 24 to 36 hours on cover glasses, they were placed in a medium with 1 μ c. of cytidine- H^3 per ml. Samples were fixed

TABLE 1
AUTORADIOGRAPHIC DATA ON INCORPORATION OF CYTIDINE- H^3 INTO RNA OF CHINESE HAMSTER CELLS IN CULTURE

Time after incorporation began	Total grains with standard errors over nucleus or cytoplasm and an equivalent area without cells (background)*			
	Nucleus	Background	Cytoplasm	Background
Strain A1290				
5-6 min.	57.0 $\pm$ 2.1	2.0 $\pm$ 0.4	3.0 $\pm$ 0.3	4.0 $\pm$ 0.3
10 min.	86.3 $\pm$ 2.1	2.9 $\pm$ 0.4	3.3 $\pm$ 0.3	6.4 $\pm$ 0.6
10 + 10 min.	77.0 $\pm$ 1.8	2.9 $\pm$ 0.8	5.2 $\pm$ 0.4	5.0 $\pm$ 0.5
10 + 30 min.	57.3 $\pm$ 1.7	3.4 $\pm$ 0.4	25.2 $\pm$ 1.2	7.4 $\pm$ 0.7
10 + 60 min.	{56.1 $\pm$ 2.1}† {82.5 $\pm$ 2.3}	3.0 $\pm$ 0.4	{58.6 $\pm$ 3.0}† {67.6 $\pm$ 2.3}	6.6 $\pm$ 0.8
10 + 2½ hours	{34.5 $\pm$ 2.4}† {69.6 $\pm$ 2.1}	2.4 $\pm$ 0.6	{106.1 $\pm$ 5.9}† {127.5 $\pm$ 6.4}	6.0 $\pm$ 0.5
10 + 4 hours	{25.8 $\pm$ 2.5}† {85.8 $\pm$ 3.8}	3.4 $\pm$ 0.3	{115.8 $\pm$ 8.1}† {139.5 $\pm$ 7.4}	6.9 $\pm$ 0.5
Strain 1404				
5 min.	34.9 $\pm$ 1.5	1.2 $\pm$ 0.3	2.3 $\pm$ 0.3	4.3 $\pm$ 0.7
10 min.	64.1 $\pm$ 2.4	1.8 $\pm$ 0.5	4.3 $\pm$ 0.4	6.1 $\pm$ 0.6
10 + 10 min.	65.0 $\pm$ 3.3	0.8 $\pm$ 0.3	6.9 $\pm$ 0.4	3.4 $\pm$ 0.4

* Average number of grains over 50 cells and background count over 10 areas of the same average area as the nucleus or cytoplasm.

† Since the amount of labeled DNA in the nucleus raises the count considerably after one half hour, the cells were divided into two classes: the 56 per cent with the lowest counts and the remaining 44 per cent (number estimated to be in DNA synthesis during the 10-min. contact).

after 5 to 6 and 10 min. in an alcoholic Bouin's fluid. The remaining cultures were rinsed in an isotope-free medium with excess unlabeled cytidine and uridine added. They were then transferred to another dish of similar medium for the remaining period of culture. All of these media were conditioned by having cells growing in them for 18 to 24 hours before use, so that uninterrupted growth would be maintained. Samples were fixed at intervals, autoradiograms prepared and the grain counts over nuclei and cytoplasm are shown in TABLE 1 and interpreted in FIGURE 4. Samples of the cells were also fixed in alcohol:acetic acid (3:1), but the autoradiograms indicated that some of the RNA leaks out of the cells with this fixative. Therefore, grain counts are given only for the material fixed in alcoholic Bouin's fixative that would be expected to precipitate and bind all polynucleotides in the cell.

The data (FIGURE 4) indicate that incorporation begins immediately in the nucleus and continues at a nearly linear rate for the 10 min. of contact. After removal and transfer of the cells to a nonradioactive medium, the dilution of precursors must be very rapid, for the grain counts at 10 min. after removal were the same or slightly lower than those for cells fixed at the time of removal. From this information one may conclude that the population of cells have labeled RNA only in the nucleus and that the total amount of labeled RNA does not significantly increase after removal of the cell from medium with cytidine- H^3 . Any change in total grain counts per cell will be due to changes



FIGURE 3. An autoradiogram showing cells treated as those in FIGURE 2, but after 10 min. the cells were washed and transferred to a medium free of cytidine- H^3 and with an excess of unlabeled cytidine and uridine for four hours. More than one half of the cells have all of the tritium in the cytoplasm where it is still incorporated in RNA. The other cells have essentially all of the labeled RNA in the cytoplasm but some tritium remains in the nucleus (lower right) of those cells that incorporate derivatives of the cytidine- H^3 into DNA.

in position of the label within the cell. The prediction would be made that as the label moves into the thin layers of cytoplasm, the efficiency of the low energy beta radiation from tritium will increase because there is less overlying shielding material. Therefore the grain counts will rise with time even though the amount of labeled RNA remains constant. Unfortunately a quantitative estimate cannot be given, for several parameters are unknown in the cells. For example, they are thickest at the nucleus and from its borders the cytoplasm tapers out to an extremely thin sheet. Usually a thin sheet of cytoplasm extends over the upper surface of the nucleus, but in some preparations this sheet bursts during fixing and drying and the naked nucleus is exposed to the film (FIGURE 3).

For the above reasons the redistribution of the label cannot be followed in

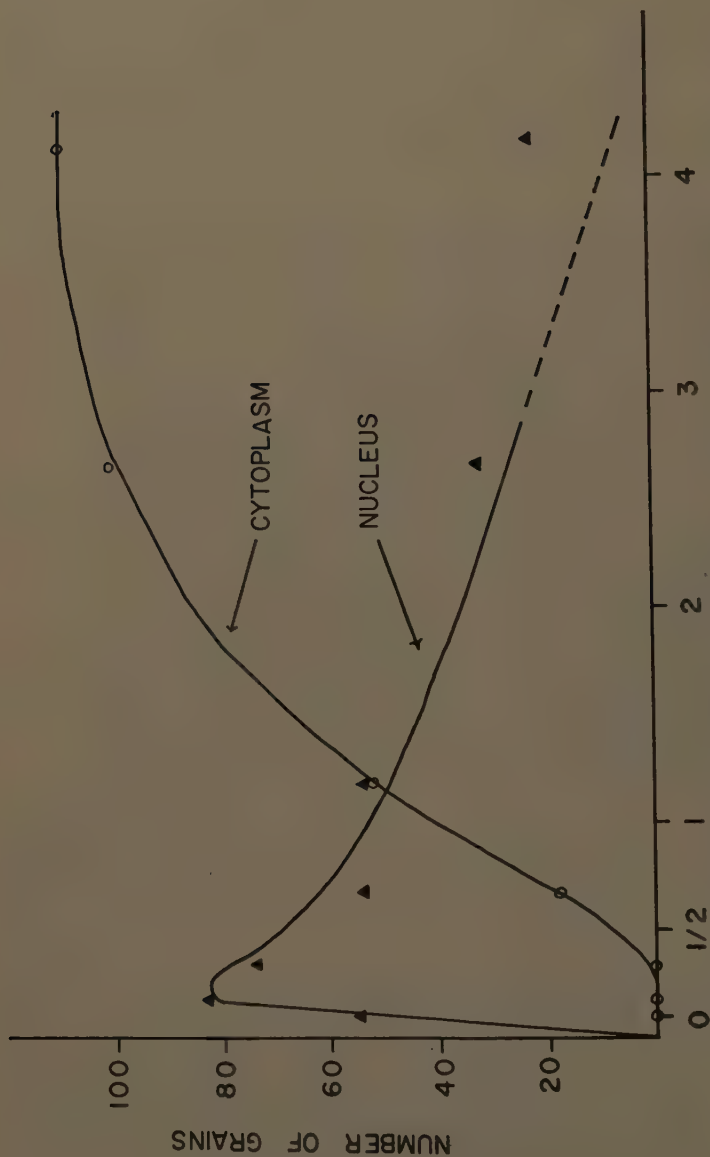


FIGURE 4. Graph showing the transfer of the labeled RNA from the nucleus to the cytoplasm in cells treated as those shown in FIGURE 3. Since the intracellular concentrations of RNA may be assumed to remain constant during the interval, the plotted points are comparable to relative specific activities.

an accurate quantitative analysis. Nevertheless the changes in geometry are not sufficient to completely distort the picture. By 10 plus 30 min. a measurable amount of the label has moved into the cytoplasm, but the total grain count per cell has not increased significantly. This might lead one to conclude that a turnover of a small fraction of the RNA is occurring that compensates for the expected increase in grain counts. By 10 plus 60 min. the expected increase in grains per cell is evident (TABLE 1). The increase was observed up to 4 hours, but the rate decreases between $2\frac{1}{2}$ and 4 hours when most of the labeled RNA has moved into the cytoplasm. All during this period as the grains over the cytoplasm increase, the labeled RNA in the nucleus decreases at the rate expected if it were contributing the label that now appears in the cytoplasm. Indeed, since there is no additional labeling indicated, the nucleus is the only source for labeled RNA. In the counts of cells at random over the slides about 43 per cent (FIGURE 1) will be selected that were in the period of DNA synthesis during the contact with cytidine- H^3 . In plotting the data in FIGURE 4 account of this was taken by separating the cells into two classes (TABLE 1), those with high nuclear counts and those with low nuclear counts. Only the 56 per cent with the lowest nuclear counts were used to arrive at the points for 10 min. plus 1, $2\frac{1}{2}$, and 4 hours. A further correction should be made for the nuclei at 2 and 4 hours. By that time nearly all of the labeled RNA had moved out of the nucleus (FIGURE 3) but because many of the cells have overlying cytoplasm the count over the nucleus would be higher than its relative content of radioactivity. The dotted line in FIGURE 4 is drawn to take this into account. Since nearly all of the labeled RNA has moved out of the nucleus in 4 hours and little if any reincorporation has occurred, there is no considerable amount of RNA that is broken down into a pool of precursors that are not diluted by the cytidine and uridine from the medium. However, there is an increase in labeled DNA with time (TABLE 1). Cells digested with ribonuclease after 10 min. contact with cytidine- H^3 show very little labeled DNA. When these are removed to the medium with an excess of unlabeled cytidine and uridine, the amount of label in DNA increases so that a readily detectable amount is seen in the cells at 1, $2\frac{1}{2}$, and 4 hours after contact with cytidine- H^3 . This would be predicted if some of the labeled pyridines are transferred into the pool of deoxyribotides, and if this pool is much less effectively diluted by the excess cytidine and uridine than the pool of labeled ribotides.

Altogether the above information indicates that essentially all of the RNA of these cells is produced in the nucleus and that it moves to the cytoplasm in a form that cannot be diluted by the pool of unlabeled pyrimidine derivatives. Although there is no direct evidence for a breakdown and loss of part of the labeled RNA, such a turnover of a fraction with a short half-life cannot be excluded from these experiments because of the changing geometry of the intracellular RNA with respect to the autoradiographic emulsion.

The role of the nucleolus in RNA metabolism is difficult to determine in these cells. Labeled RNA appears very soon in the nucleolus, but at the same time it is distributed throughout most of the nucleus. In one strain of cells ($\phi 1404$) the nucleoli are rather discrete and there are only a few in each cell.

On the other hand in the strain (σ^7 A1290) there appear to be more regions on the chromosomes where nucleolar material collects and large nucleoli are not usually visible. In the early prophase cells of strain 1404, most of the labeled RNA is localized in nucleoli, but in the other strain the RNA labeled during the preceding 10 to 20 min. is more uniformly distributed over the chromosomes. When the cells are treated with colchicine during the incorporation and the succeeding period, up to 1 or 2 hours, much of the labeled RNA remains associated with the chromosomes in the colchicine blocked metaphases. In the absence of colchicine, however, most of the labeled RNA has dispersed into the cytoplasm before metaphase. Then during the reorganization of the nuclei at telophase, relatively little of the labeled RNA was included in the nuclei. There was variation among nuclei and sometimes a detectable amount of the label that remained nearby appeared to be included in the reorganizing nucleus as if by accident.

TABLE 2
RATIOS OF CELLS AT VARIOUS STAGES OF DIVISION WITH OR WITHOUT LABELED RNA (LABELED:UNLABELED) AT VARIOUS INTERVALS AFTER INCORPORATION OF CYTIDINE- H^3 BEGAN

Stages of division	5 min.	10 min.	10 + 10 min.*	10 + 30 min.*	10 + 60 min.*
Early prophase	15:8	35:0	25:0	28:0	32:0
Mid-prophase	13:7	17:4	15:0	19:0	14:0
Late prophase	1:30	11:12	19:4	24:0	16:0
Metaphase	0:49	0:50	35:23	54:2	29:1
Anaphase	0:34	0:26	6:36	28:0	28:1
Early telophase	0:12	0:12	0:23	17:1	14:0
Late telophase	18:19	25:12	9:9	27:0	23:0

* Ten minutes in a medium with cytidine- H^3 with the additional period in the medium with an excess of unlabeled cytidine and uridine.

Discontinuities in RNA synthesis associated with mitosis. Incorporation of cytidine- H^3 occurred at all stages in interphase and in mitosis until about mid-prophase. As the chromosomes became condensed the synthesis of RNA stopped (TABLE 2 and FIGURE 2) even though the nucleoli were still present. This observation might be used as evidence that the expanded chromosomes are essential for RNA synthesis, for during about 30 min. from mid-prophase to mid-telophase there is no synthesis of RNA detectable by incorporation of cytidine- H^3 . This is not due to the failure of the dividing cell to take up and incorporate materials from the medium, for arginine- H^3 is incorporated into proteins of the cytoplasm at about the rate characteristic of interphase stages.

Another difference between the behavior of RNA in prophase and interphase is the rate at which it moves to the cytoplasm. The movement is faster in late prophase. The more rapid dispersion may be associated with the breakdown of the nuclear membrane. In the cells fixed 30 min. after removal from the medium with cytidine- H^3 , less than one third of the label had moved to the cytoplasm in cells at interphase (TABLE 1). However, if the cells were at late prophase or metaphase when fixed, most of the labeled RNA was already in the cytoplasm. As mentioned above, an exception was found when the cells were treated with colchicine.

Colchicine was utilized in another way to find whether synthesis or turnover of RNA could be detected in the cytoplasm after longer periods of contact than from mid-prophase to mid-telophase. Cells were placed in a medium with colchicine for 2 hours and then in a medium with both colchicine and cytidine- H^3 for 2 hours. When the cells were fixed and autoradiograms prepared, about 50 per cent of the cells blocked at division were labeled and about 50 per cent were almost unlabeled. Those with label were cells that had reached division during the 2 hours in cytidine- H^3 and therefore had a chance to incorporate label before the chromosomes condensed. Those without label had been blocked during the two hours before being placed in the medium with cytidine- H^3 . Therefore during the whole period in contact with label, the chromosomes had been supercondensed according to the reaction typical with colchicine.

Cells that were in isotope for 2 hours with colchicine contained a trace of label, perhaps 2 to 3 per cent of the amount present in interphase cells. No tests were performed to show that the trace of label was in RNA, but in alcohol-acetic acid fixed cells it did not appear to be retained. Perhaps it was a soluble RNA or protein-bound nucleotide. The principal result is clear; although colchicine has no effect on synthesis of RNA in interphase cells, it prevents synthesis in cells by holding the chromosomes in a condensed metaphase state for prolonged periods. The uptake and incorporation of arginine- H^3 into the cytoplasm of cells held in division by colchicine was little if any diminished during a two-hour period with conditions similar to those used for the experiments on cytidine incorporation.

The data recorded in TABLE 2 show the interruption of RNA synthesis associated with division and also provide a measure of the duration of some of the division stages. After 5 min. contact with cytidine- H^3 early and mid-prophases and late telophases were labeled. By 10 min. late prophases but no metaphases were labeled. Therefore synthesis of RNA ceases 10 min. or more before metaphase. Within another 10 min. nearly all metaphases and a few anaphases were labeled. No early telophases were labeled, but 40 min. after contact with cytidine- H^3 all anaphases and nearly all early telophases were labeled. Therefore, the duration of all stages from mid-prophase to mid-telophase is about 30 min.

Discussion

Tritium-labeled nucleosides of high specific activity and cells in culture provide favorable materials for studying short term incorporation. This allows an analysis of the sequence of duplication of the chromosomes, for the events in 5 to 10 min. of a DNA synthetic period of about 6 hours can be seen. The analysis clearly shows that not all parts of the chromosome complement are duplicated simultaneously. The sequence of duplication is a strain characteristic, but since only two strains, one isolated from a male and one from a female embryo, have been examined generalizations should be made with caution. However, the different timing in duplication of the long arm of the two X chromosomes in the female suggests that heterozygosity with respect to duplication time may exist (Taylor, article in press).

The experiments show that dilution of the thymidine- H^3 in the cells at the

concentrations used is almost instantaneous. Once incorporated into DNA, however, the tritium is conserved and equally distributed to the daughter cells.

Similar experiments with cytidine- H^3 as the source of label reveal that the labeled precursors can be diluted quite rapidly but the quantitative aspects of the study do not exclude a turnover of a fraction of the RNA. Cytidine- H^3 is rapidly deaminated in higher plant cells (Taylor, 1959b) and in HeLa cells (Taylor, unpublished) so that uracil and, to a lesser extent, thymine-containing nucleotides are labeled. Addition of an excess of unlabeled uridine and cytidine can rapidly dilute the pool of labeled nucleotides so that increase in labeled RNA was prevented in these Chinese hamster cells. However, this conclusion had to be based on evidence collected over a very short time interval. As soon as significant amounts of labeled RNA moves from the nucleus to the cytoplasm, the geometrical relations of film and site of radioactive material are changed and further quantitative comparisons cannot be made. Since no increase in labeled RNA occurred during the first 10 min. interval after removal from the medium with isotope, one may conclude that the precursors were effectively diluted by the excess cytidine and uridine. After this initial period, the total grain count per cell increased, but this increase would be predicted without a net increase in labeled RNA if it moves from the nucleus to the cytoplasm.

Failure to take this change into account may have led Harris (1959) to reach a different interpretation of autoradiographic data on cells in culture. Harris assumed that no change in efficiency would occur and therefore considered the increase in grains to be due to incorporation of soluble precursors that could not be diluted by excess uridine and cytidine from the medium. Although independent evidence indicated that the specific activity of the RNA continued to increase, the autoradiographic data probably reflect both changes, the actual increase and the increase due to change in geometry. In his experiments the grains over the nucleus did not begin to decrease immediately as the cytoplasmic labeling rose. Although he reached the conclusion that nuclear and cytoplasmic synthesis were independent events, his data are compatible with the hypothesis that all RNA is formed in the nucleus. The residual precursors, if only utilized for synthesis of RNA in the nucleus followed by transport to the cytoplasm, would have kept the grain count over the nucleus from dropping immediately.

Since in the Chinese hamster cells there is no increase in labeled RNA after the 10-min. labeling period, all of the label that later appears in the cytoplasm must have come from the nucleus. If there were an appreciable turnover of RNA, the amount of labeled RNA in the nucleus would not have fallen to such a low level as shown in FIGURE 3 because the concentration of RNA in the nucleus is as high or higher than in the cytoplasm. From these experiments no quantitative measure of turnover can be given because the grain count increases during the four-hour period studied. A small amount of turnover could have been compensated for by the change in geometry. However, there is no evidence indicating that a measurable turnover occurs in cells in log-phase growth. Indeed the evidence is on the other side. Siminovitch and Graham (1956) found none and the data of Harris (1959) indicate that connective tissue

cells in culture show no turnover between 4 and 12 hours after incorporating adenosine- H^3 into RNA. Harris found a turnover in macrophages in cultures that were not growing. Perhaps in these most of the label was incorporated in the soluble RNA which may turnover during protein synthesis, while in rapidly growing cells most of the label is incorporated into RNA of ribosomes.

Summary

Nucleic acid metabolism was studied with cells of Chinese hamster in culture by use of tritium-labeled nucleosides and autoradiography. Chromosomes of a single complement were shown to duplicate asynchronously. Difference in timing occurred among the different chromosomes and within a single chromosome. The patterns of duplication are a strain characteristic, but vary in the two strains examined.

RNA synthesis was shown to be confined to the nucleus in these cells. Incorporation of nucleosides was continuous in the rapidly growing cells except that it stopped when the chromosomes were condensed during division. In the same cells protein synthesis as measured by amino acid incorporation in the cytoplasm was unaffected by the division stage. After cells incorporate cytidine- H^3 for only 5 to 10 min. all of the labeled RNA is in the nucleus. By washing and removing the cells to a medium free of labeled nucleosides and with excess unlabeled cytidine and uridine further incorporation of the labeled precursors in RNA could be effectively stopped. The labeled RNA of such cells is nearly all found in the cytoplasm after about four hours.

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DYNAMIC ASPECTS OF MITOTIC APPARATUS PROTEIN*

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The magnificent observations of the classic cytologists during the latter half of the last century on dividing cells were frequently accompanied by a discussion of the possible origin of the achromatic figure (Boveri, 1887; Strasburger, 1900; Wilson, 1925). This was not pure speculation, but was based upon the cytological observations. Generally the mechanism of formation of the achromatic figure was considered in terms of the rearrangement of pre-existing subcellular structures visible in the cytological preparations. Indeed, the assembly of this structure apparently was not considered in any other terms. Thus, in a broad sense, the "precursor concept" of the origin of the achromatic figure arose shortly after the initial observations established its presence in dividing cells. After about fifty years of relative dormancy, the precursor concept of the origin of the achromatic figure is being revived and put to more direct experimental test. The method for isolating the mitotic figure from cleaving sea urchin eggs (*Strongylocentrotus purpuratus*) was developed by Mazia and Dan (1952). This permitted the formulation and execution of experiments designed to test directly the origin of the mitotic apparatus, which is a complex of the achromatic figure and the chromosomes. It was imagined that the definitive mitotic apparatus could arise either as the result of the *de novo* synthesis from such small units as amino acids or small polypeptides or as the result of the assembly of preformed macromolecular subunits present in the cytoplasm. The latter concept, the precursor concept, differs from the earlier views only at the level of structural complexity. The early cytologists thought in terms of the rearrangement of microscopically detectable structures, while this paper views the precursor units as being of macromolecular dimensions. The precursor concept predicts the existence in the unfertilized egg of molecules identical to those responsible for the structural continuity of the mitotic apparatus. On the other hand, if the *de novo* synthesis of the mitotic apparatus were the mechanism employed by the cell, one could reasonably expect its constituent molecules to be foreign to the unfertilized egg. The experimental results, therefore, should permit a choice of either alternative to the exclusion of the other. It was felt that immunochemical procedures could yield results that would allow such a decision to be made. Accordingly antisera were prepared in rabbits against unfertilized egg antigens (anti-egg serum) and against solutions of dissolved mitotic apparatus (anti-DMA) isolated by the digitonin method (Mazia and Roslansky, 1956). Provided with antibodies against two basically different systems—the unfertilized egg and the fertilized egg that has embarked upon its predetermined course of development—antigen solutions from various sources could be compared immunochemically. The Ouchterlony gel diffusion technique (Oudin, 1952; Ouchterlony, 1958) was employed for this purpose because of its high resolv-

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ing power of multiple antigen-antibody systems and the sensitive criterion it provided for establishing an identity or nonidentity between two antigens from different sources. One could now compare, immunochemically, an extract of unfertilized eggs to a solution of dissolved mitotic apparatus. A detailed presentation of experimental procedures and of these results has been given elsewhere (Went, 1959), and they will be considered only briefly here. These procedures and results reveal that a solution of mitotic apparatus isolated by the digitonin method (Mazia and Roslansky, 1956) reacts with antiserum prepared against unfertilized egg antigens and with its homologous antiserum to form only a single band of precipitate in the agar, and that this fused (interfered) with a band assignable to unfertilized egg antigens. This fusion establishes an immunochemical identity between the responsible antigens. It should be mentioned that an identity between two proteins based solely upon the fusion of two bands in an Ouchterlony plate must be restricted within the limitations, not completely understood, inherent in the procedure itself. The data establish the presence in the unfertilized egg of a molecular species, termed the precursor-1 component, identical to an antigen invariably found in all solutions of dissolved mitotic apparatus investigated, and the simplicity of the precipitation patterns substantiates earlier suspicions (Mazia, 1959) that the mitotic apparatus is molecularly relatively homogeneous. This qualitatively supports the precursor concept for the origin of the mitotic apparatus, for the evidence demonstrates that the molecular constituents of the mitotic apparatus are on hand in the unfertilized egg prior to the initiation of the mitotic cycle. That is, the cell apparently does not have to synthesize a new molecular species in order to construct the mitotic apparatus although quantitative determinations are required before it is known whether the mitotic apparatus can be assembled to completion from the amount of precursor-1 component on hand in the unfertilized egg or whether additional material must be synthesized. Another inference can be made from the observations. The process of dissolving the mitotic apparatus, in terms of the end products observed, may be considered a reversal of the assembly process in view of the consistent appearance of the precursor protein in solutions of dissolved mitotic apparatus. This implies that the intermolecular bonds responsible for linking the precursor protein molecules to each other to form the definitive mitotic apparatus are considerably more labile than are the intramolecular bonds of the precursor protein molecules themselves—a behavior compatible with the view that the mitotic apparatus represents a reversible aggregation of precursor molecules.

Earlier determinations (Mazia and Roslansky, 1956) had shown that the cleaving sea urchin egg invested about 12 per cent of its total protein in the metaphase mitotic apparatus. If all of the precursor protein is on hand in the unfertilized egg there might be a basis for optimism that one could isolate it in quantity from unfertilized eggs, which would allow performance of otherwise nonfeasible experiments. Already before the immunochemical evidence for the existence of the precursor protein was available, the presence of a protein sensitive to calcium ions had been established in the soluble fraction of a 0.1 *M* KCl extract of unfertilized sea urchin eggs. When calcium chloride was added to such an extract to a final concentration of 0.05 *M*, there appeared a clear, fibrous

precipitate that could be put back into solution by dialysis against distilled water. This calcium-insoluble protein could be obtained in a very homogeneous form (Kane and Hersh, 1959) and comprised about 10 per cent of the total protein of the unfertilized egg. Physical and chemical characterization studies by Kane and Hersh on this protein revealed a rather strong similarity between it and the protein of isolated mitotic apparatus. They shared similar molecular weights, each contained a ribonucleotide component and both were relatively asymmetrical molecules. Since the calcium-insoluble form could be obtained in a very homogeneous form (by ultracentrifugal standards) it was a simple matter to establish whether or not it was immunochemically identical to the precursor-1 component. The bands resulting from the interaction of the calcium-insoluble protein and the dissolved mitotic apparatus with antiserum to unfertilized egg antigens intersected, indicating a nonidentity between these proteins. This was further corroborated with an absorption experiment in which material that could absorb the antibodies specific for the precursor-1 component from the antiserum did not react with the antibodies specific for the calcium-insoluble fraction. Thus the attractive possibility that this fraction could be the precursor-1 component was demolished.

As long as only solutions of mitotic apparatus isolated from preserved material by the digitonin method were available, gel diffusion analysis of these by numerous antisera prepared against various solutions and suspensions of unfertilized egg antigens, and an antiserum prepared against dissolved mitotic apparatus revealed that only the precursor-1 component was invariably present.

Periodically another band of precipitate appeared, signifying the presence of another antigen. Recently a method has been developed that allows one to isolate the mitotic apparatus directly from living material (Mazia, 1959). That is, the material was alive at the instant it was exposed to the medium used for isolating the mitotic apparatus. The mitotic apparatus isolated by the new method could be put into solution under milder conditions than those necessary for digitonin-isolated mitotic apparatus. A distilled water suspension of the latter could be dissolved usually only at pH 10.5, while the mitotic apparatus isolated directly from living material were soluble in distilled water at pH 8.5-9. When solutions of mitotic apparatus isolated from living material were subjected to close scrutiny by gel diffusion methods 2 bands of precipitate were consistently observed. One of these was the familiar precursor-1 component band, but the other one could not be identified with any known antigen. However, like the precursor-1 component, the antigen responsible for the other band was common to the unfertilized egg. This antigen has been termed the precursor-2 component pending clarification of its structural affinities with the mitotic apparatus. As a routine procedure, antiserum was prepared against dissolved mitotic apparatus isolated from living material. When this was tested against various antigen solutions it became abundantly apparent that this antiserum (anti-FMA) could not react visibly with the precursor-1 component, although the immunizing solutions were known to contain considerable quantities of this antigen, but could react only with the precursor-2 component. That is, the anti-FMA was absolutely specific for the precursor-2 component. The question then arose—why could the precursor-2 component

only infrequently be detected in solutions of mitotic apparatus isolated by the digitonin method? Two alternatives suggested themselves: (1) the precursor-2 component may have been removed during the isolation procedure, and (2) the ability of the precursor-2 component to combine with its homologous antibody may have been destroyed by the elevated pH used to put the digitonin-isolated mitotic apparatus into solution, while the same treatment did not affect the precursor-1 component. The second alternative was put to experimental test, and the results clearly indicated that the combining effectiveness of the precursor-2 component could be nearly completely destroyed by the conditions used to dissolve the digitonin-isolated mitotic apparatus although it was unaffected by the procedure used to dissolve the mitotic apparatus isolated from living material. From the data presented so far it was not possible to make any decisions regarding the importance of the precursor-2 component to the structural continuity of the mitotic apparatus. In an attempt to establish the relative importance of the two precursor components to the mitotic apparatus some preliminary fluorescent antibody staining experiments were conducted. The first cleavage prophase to anaphase stages were prepared for staining with fluorescent antibodies by the freeze-substitution method described by Woods and Pollister (1955) modified according to suggestions of B. J. Davis (personal communication). This method was selected as probably the least likely to destroy the combining properties of the antigens of interest, while retaining any soluble antigens in a relatively native cytological orientation. The indirect method of fluorescent-antibody staining was used (Weller and Coons, 1954). That is, the material was made to react with either anti-DMA or anti-FMA after which it was allowed to react with antirabbit globulin serum conjugated with a fluorescent dye, 5-dimethylamino-1-naphthalene sulfonyl chloride (Wolochow, 1959; Chadwick *et al.* 1958). The results were rather surprising. Those embryos that were made to react with the anti-FMA serum (specific for the precursor-2 component) displayed uniform fluorescence of the cytoplasm at an intensity somewhat greater than that of the negative fluorescence controls, while the material that was made to react with the anti-DMA serum exhibited brightly fluorescent areas roughly equal in size and shape to the mitotic apparatus contrasting sharply with the lower intensity fluorescence of the surrounding cytoplasm. It would appear that the precursor-1 component is intimately associated with the mitotic apparatus, but still no definite conclusions regarding the role of the precursor-2 component in the structural continuity of the mitotic apparatus can be drawn owing to the negative aspect of the results. Although one would not expect to lose water-soluble proteins during the freeze-substitution procedures nor subsequent to fixation, it remains to be demonstrated that no loss of antigenic material occurred. Mitotic apparatus isolated by the digitonin method can be shown to react with anti-DMA by means of the fluorescent-antibody technique.

The results described above are consistent with the precursor concept of the origin of the mitotic apparatus. Not only does the unfertilized egg contain the molecular species found in the mitotic apparatus, but the completely formed mitotic apparatus appears to contain no molecular species not found in the unfertilized egg. Thus it can be imagined that the mitotic apparatus resides

unassembled in the unfertilized egg and that the entry of a spermatozoan initiates, among many others, processes responsible for the assembly of the precursor molecules into the definitive mitotic apparatus.

Experimental inquiry into the intracellular distribution of the precursor components could be expected to yield pertinent information. Presently we have been dealing either with dissolved mitotic apparatus or the soluble fraction of a 0.1 *M* KCl extract of ethanol-preserved unfertilized sea urchin eggs (Went, 1959). By means of absorption experiments it was established that the particulate fraction of unfertilized eggs also contains antigens capable of combining specifically with the antibodies homologous for the precursor-1 component. The particulate fraction, which is arbitrarily defined as everything that can be sedimented at 60,000 g for 30 min. from a 0.1 *M* KCl homogenate of ethanol-preserved eggs, was extracted by various procedures (Went, 1959) and subsequently used to absorb antiserum containing antibodies specific for the precursor-1 component. The results revealed that tenaciously associated with the particulate fraction was an antigen that could combine specifically with antibodies homologous for the precursor-1 component. This is presumably the precursor-1 component in an "insoluble" form. Living material was then studied in an attempt to determine if the precursor components were confined within certain cytoplasmic particles known to behave as osmometers (Harris, 1943), or are present in the surrounding cytoplasm, or both. The living eggs were gently dispersed in an isotonic dextrose medium in a manner that avoided disrupting the osmotically active particles. The homogenate was then centrifugally fractionated roughly into three fractions: (1) the osmotically active particles, called yolk particles; (2) the microsome-mitochondria fraction; (3) and the supernatant to the microsome-mitochondria fraction. The yolk particles were ruptured osmotically after careful washing and the lysate separated from the solid material. The solid material was then extracted in 0.5 *M* KCl and the supernatant termed the lysed yolk particle extract. The microsome-mitochondria fraction was also extracted in 0.5 *M* KCl. The 4 resultant solutions—yolk particle lysate, supernatant to the microsome-mitochondria fraction, lysed yolk particle extract, and the extract of the microsome-mitochondria fraction were analyzed by the Ouchterlony gel diffusion technique. The precursor-1 and precursor-2 components were found in all 4 fractions. However, 2 fractions, the lysed yolk particle extract and the microsome-mitochondria extract, yielded solutions in which the 2 precursor components were the only or major antigens detectable. Undoubtedly other protein species were present, but escaped detection since the antiserum did not contain bodies specific for them. The general picture that emerges is that both precursor components are found within the yolk particles, free in the surrounding cytoplasm, and in association with the structural aspects of various subcellular particles.

The fact that the precursor-1 component occurs not only as a soluble and an insoluble antigen, but also that it is the only antigen bearing an intimate structural relationship to the definitive mitotic apparatus, opens the door to the realm of speculation. One can reasonably hypothesize that there exists a dynamic equilibrium between the soluble and insoluble forms of the precursor-1 component. The insoluble fraction would represent the major pool of this

protein, being converted into the soluble form as an intermediate before its incorporation into the developing mitotic apparatus. This implies that one might expect the soluble precursor-1 component level to remain more or less constant during the formation of the mitotic apparatus, and it may or may not be exhausted when the mitotic apparatus is fully formed. FIGURE 1 presents a hypothetical scheme for the incorporation of precursor-1 component molecules into the mitotic apparatus consistent with experimental data. Experiments have indicated that the embryo at the time of the first cleavage division contains an amount of soluble precursor-1 component comparable to to the level in the unfertilized egg. If this last observation is valid, then the precursor-1 component incorporated into the mitotic apparatus must have come from the insoluble pool, provided that there had not been any net synthesis of this protein. These data themselves really do not produce a clear image

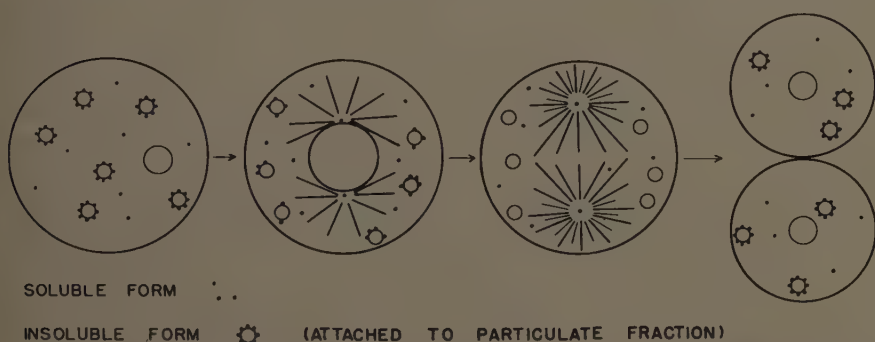


FIGURE 1. This is a diagrammatic representation of a hypothetical scheme for the reversible incorporation of insoluble precursor protein molecules into the developing mitotic apparatus by way of a soluble intermediate stage. The small dots denote the precursor molecules, either attached to larger subcellular structures (insoluble form) or free in the cytoplasm (soluble form).

of events during the formation of the mitotic apparatus, but do allow one to consider the mitotic apparatus as a reversible aggregation of precursor molecules that themselves remain structurally unmodified during these transitions.

Is the precursor-1 component unique to cells in active division and to those preparing for division, or can it also be found in cells normally destined to undergo no further mitoses? One cannot presuppose the absence of mitotic apparatus precursor molecules from differentiated cells, for they may perform a dual function in the over-all economy of the cell, consequently being retained for other purposes upon the termination of normal mitosis. However, if the precursor molecules uniquely serve in the structural continuity of the mitotic apparatus, then they may well be absent from cells neither dividing nor preparing for division, in which event it should be possible to identify cells preparing for division in a population of nondividing cells. Various embryonic and adult tissues were analyzed for the presence or absence of the precursor-1 component (Went, 1959; Went and Mazia, 1959). The blastula and prism larva stages contain the soluble form of the precursor-1 component in amounts similar to that found in the unfertilized egg. This was determined by the

Ouchterlony gel diffusion method and it is realized that 100 per cent variation in concentration of the precursor-1 component is about the limit of sensitivity of the quantitative determinations carried out by this method. However, the observed variation in precursor-1 component level during development is much less than would be expected if a strict correlation existed between the number of mitoses microscopically detectable and the precursor-1 component level. The adult tissues tested include ovary, testis, gut, and lantern muscle. The precursor-1 component could be detected only in the extract of ovaries, being absent or below detectable levels in the other tissues examined. The results with the ovaries and testes are especially interesting. Feulgen-stained sections of gonad from the animal whose other gonads furnished the antigen were carefully observed. In neither the ovary nor the testis were mitotic or meiotic figures visible, but the ovary contained some mature ova. The presence of the precursor-1 component antigen in the extract of ovary can be accounted for by the occurrence of the mature ova. In summary, only tissues in active mitoses or preparing for division contain detectable levels of the precursor-1 component.

Although the evidence presented is neither inconsistent with the precursor concept for the origin of the mitotic apparatus nor with the view that the precursor material remains on hand after division if, during the normal subsequent sequence of events, the cell is soon to divide again, other plausible interpretations suggest themselves. For example, the distribution of the precursor-1 component also coincides exactly with whether the tissue was embryonic or adult in origin. No precursor-1 component was detectable in adult tissues (the ovary must be excluded from this consideration), while no embryonic tissue was devoid of this antigen.

This presentation is primarily intended to direct attention to a plausible scheme for the reversible aggregation of preexisting molecular subunits into the mitotic apparatus, consistent with experimentally established facts. However, the restricted scope of the data and the specialized nature of the experimental material must be emphasized. The unfertilized egg is a specialized cell provisioned for a series of rapid cleavage divisions without alternating periods of net synthetic activity. Thus all the precursor material to the mitotic apparatus necessary to the embryo through the stage when feeding begins may be on hand before initiation of development. In the more usual situation where the cell must synthesize new material prior to division, one can expect to find active synthesis of the precursor component(s) during interphase. Finally, study of the appearance and disappearance of the precursor molecules themselves may provide important insight into processes of differentiation and regeneration.

Acknowledgments

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DYNAMICS OF SPINDLE FORMATION

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The mitotic spindle is usually not visible in living cells when observed by ordinary or phase contrast microscopy except as a clear area differentiated from the surrounding cytoplasm by the absence of granules. In polarized light, the spindle has been clearly demonstrated in a variety of living cells,¹⁻⁴ and the development of the spindle in the newt (*Trilurus viridescens*) was reported earlier.⁵

Classical cytologists have defined two types of spindles: (1) the astral, which is characteristic of the higher plants; and (2) the amphiastral, which is typical of the mitoses of higher animals generally.⁶ The amphiastral type has been further subdivided^{6,7} according to the behavior of the asters. In one class, the asters separate in early prophase and the spindle is formed between them. The cells of salamander form a well-studied example of this class. In the second class, the asters separate and take up positions on the nuclear membrane that are often polar to each other. This separation may take place in early prophase or even during the telophase or reconstruction stages of the previous division. In the few species in which a spindle has been described between the separating asters, it subsequently disappears, and in early prophase the asters are separate and unconnected. After the nuclear membrane breaks down, spindle fibers appear to condense out of the nuclear region. The eggs of a number of lower forms (the annelid-thalassema; the nemertine-cerebratulus) of white fish⁸ and of *Drosophila melanogaster*⁹ are examples of the second class. Wasserman,⁷ in his review, states that the second class is so common for metazoan cells that it could be considered the rule but for some instances of the first class.

The newt, which belongs to the first class, has been used extensively in this laboratory for studying mitosis in tissue culture but such cultures do not provide sufficient material for chemical analysis. Accordingly, human cells in tissue culture are being used for this purpose. A survey of the literature shows that much less is known of spindle formation in mammalian cells as compared with lower forms. We shall report briefly on some studies of spindle formation in human cells.

Experimental

The cells (Chang's strain derived from normal human liver) were grown on coverslips placed on the bottom of a Petri dish. The coverslips were immersed in Eagle's basal media and incubated in a moist CO₂ chamber. Before use the cover slip was transferred to a hollow slide, the bottom of which is made with another cover slip. Medium was added, leaving only a small air bubble. All operations were performed in a warm room at 37.5° C. Observations were made with a modified Leitz petrographic microscope, which has been described previously.⁵

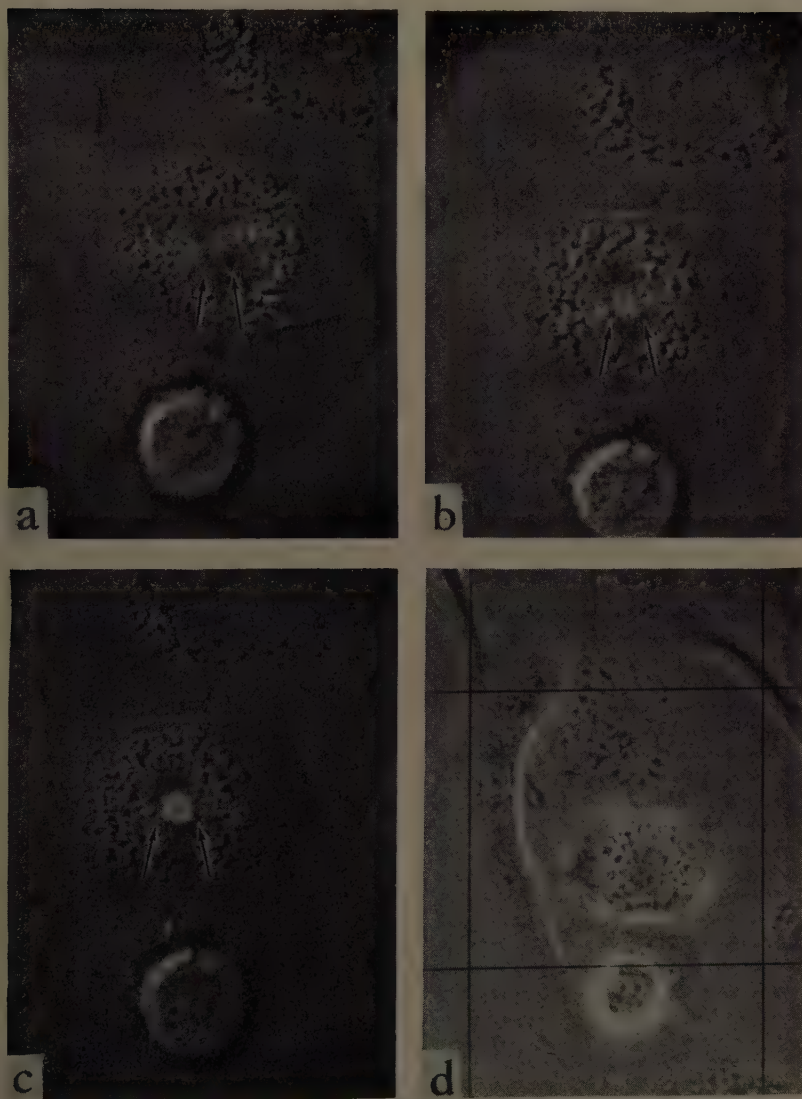


FIGURE 1. Configurations of the mitotic spindle in a human cell in tissue culture. (a) Arrows indicate asters that are just visible, with spindle between them. (b) One and one-half minutes after *a*. Spindle and asters clearly visible. (c) Three minutes after *a*. Spindle is elongating. (d) Four minutes after *a*. The same cell as seen by phase contrast.

(FIGURE 1 continued on following page)

Results

The formation of a typical spindle is shown in FIGURE 1. The earliest birefringent body that has thus far been observed consisted of two asters connected by a spindle. The length was 4 to 5 μ . In some cases the nuclear membrane appeared to be present at this stage. The astral rays were often quite long,



FIGURE 1 (*continued*). (e) Five minutes after *a*. (f) Eight minutes after *a*. Spindle has reached maximum length. (g) Twenty-seven minutes after *a*. Typical metaphase plate. Note that spindle is shorter and broader than in *f*. (h) Forty-seven minutes after *a*. Constriction.

exceeding the length of the spindle. Elongation appeared to begin at about the same time as loss of the nuclear membrane. The spindle grew to about twice its original length. This elongation was followed by a shortening and thickening phase. The length changes are illustrated by the graph (FIGURE 2). The length of the spindle reached a minimum value some time before anaphase.

Discussion

Our results to date indicate that the formation and subsequent changes in the spindle closely resemble those found in the newt, including the shortening during metaphase. Although the very earliest stages have not been seen, the

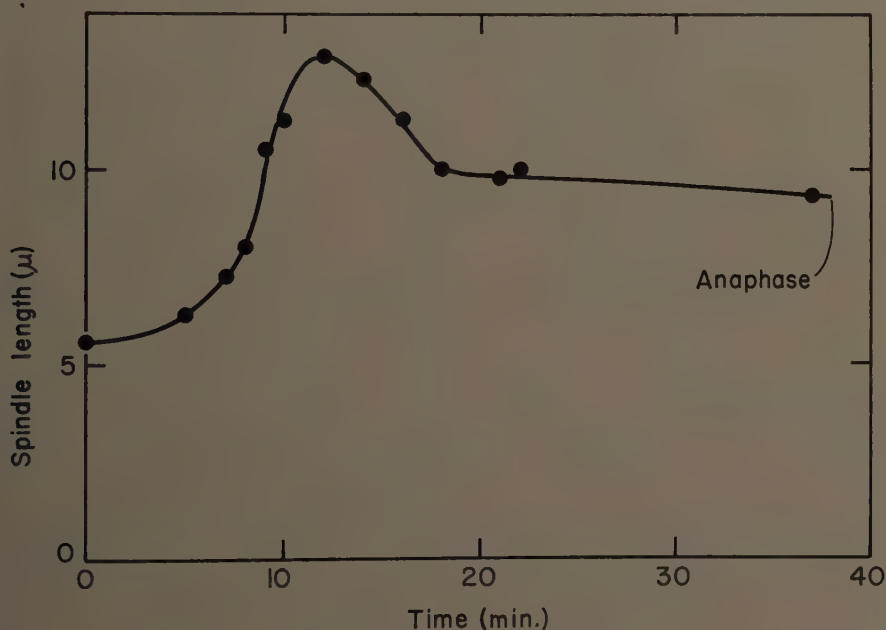


FIGURE 2. Rate of spindle formation.

configurations observed show that the human cell belongs to the same class of spindle formation as the newt. The elongation stage is less pronounced in the human cell; the spindle increases in length by a factor of 2 to 2.5, while the newt spindle increases by a factor of 5 or 6. The clearest difference between the spindles is during anaphase. In the human cell, after the chromosome masses have separated, continuous fibers are present, while in the newt cell no birefringence is detectable by our technique.

The rate of spindle elongation in both cells that have been studied is of the same order as the rate of separation of the poles in anaphase. The two processes during anaphase, namely the motion of the chromosome to the poles and the separation of the poles, need not occur by the same mechanism. The pole separation may be brought about by the same process that accounts for the growth of the spindle in prophase. This step would not be an active expansion

but a condensation of protein by the centrosomes. Some evidence that the amount of material in the spindle increases during anaphase has already been given by Rukstad.¹⁰

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DISAPPEARANCE OF SPINDLES AND PHRAGMOPLASTS AFTER MICROBEAM IRRADIATION OF CYTOPLASM

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Disappearance of Spindles

In earlier publications^{1,2} it was reported that heterochromatic ultraviolet microbeam irradiation^{3,4} of a small portion of the cytoplasm of metaphase newt (*Triturus*) cells in tissue culture caused the spindle to diminish or, if exposure was sufficient, disappear. Since spindles were thus affected even when the site of bombardment was as far as 30 μ away, it was concluded that the effect is probably mediated by a spindle "poison" produced photochemically from a precursor that is some constituent of normal cytoplasm.

It is of some interest to know whether the precursor is present only in newt cells or is a widely distributed cellular substance. As an approach to this question, experiments were performed on developing sand-dollar eggs⁵ (R. B. Uretz and R. E. Zirkle, in preparation) and it was found that in the two-celled stage, with each cell showing a bright metaphase spindle by polarization microscopy, suitable UV microbeam irradiation in the cytoplasm of one cell caused the spindle birefringence* to disappear promptly in that cell but not in the other. Thus it would appear that the precursor is some substance present both in vertebrates and in echinoderms.

Recently these observations have been extended to the seed plants (R. H. Haynes and R. E. Zirkle, in preparation) by use of the Bajer preparation of endosperm cells of *Haemanthus Katharinae*.⁶ A typical result, as observed by polarization microscopy, is shown in FIGURE 1. A slightly flattened metaphase cell is shown in FIGURE 1a, the spindle axis extending vertically. The spindle is bright by polarization microscopy. In FIGURE 1b the site of bombardment is indicated by the circumscribing cross-hairs (phase microscopy). FIGURE 1c was taken 7 min. after irradiation; the birefringence of the spindle is no longer visible. It therefore appears that the precursor probably has a wide distribution among taxonomic groups.

Effects on Phragmoplasts

By polarization microscopy, the plant spindle disappears during anaphase, and afterward the phragmoplast develops in the interzonal region; like the spindle, it is strongly birefringent (FIGURE 2a). In terms of the poison hypothesis of spindle disappearance, it is of interest to determine whether the phragmoplast responds as does the spindle to UV irradiation of cytoplasm.

The answer to this question is affirmative, as shown in FIGURE 2. FIGURE 2a shows a cell before irradiation; the compensator was adjusted to make the birefringent phragmoplast appear dark. FIGURE 2b (phase microscopy) shows the site of irradiation near the end of the cell; 2c, taken 8 min. after irradiation, shows the birefringence nearly gone; 2d was taken 14

* The birefringence of all spindles and phragmoplasts discussed in this paper is positive with respect to the spindle polar axis.

min. after irradiation, at which time the birefringence had completely disappeared. This similarity in response of spindle and phragmoplast would tend to support the long-standing notion that these birefringent structures are architecturally related.

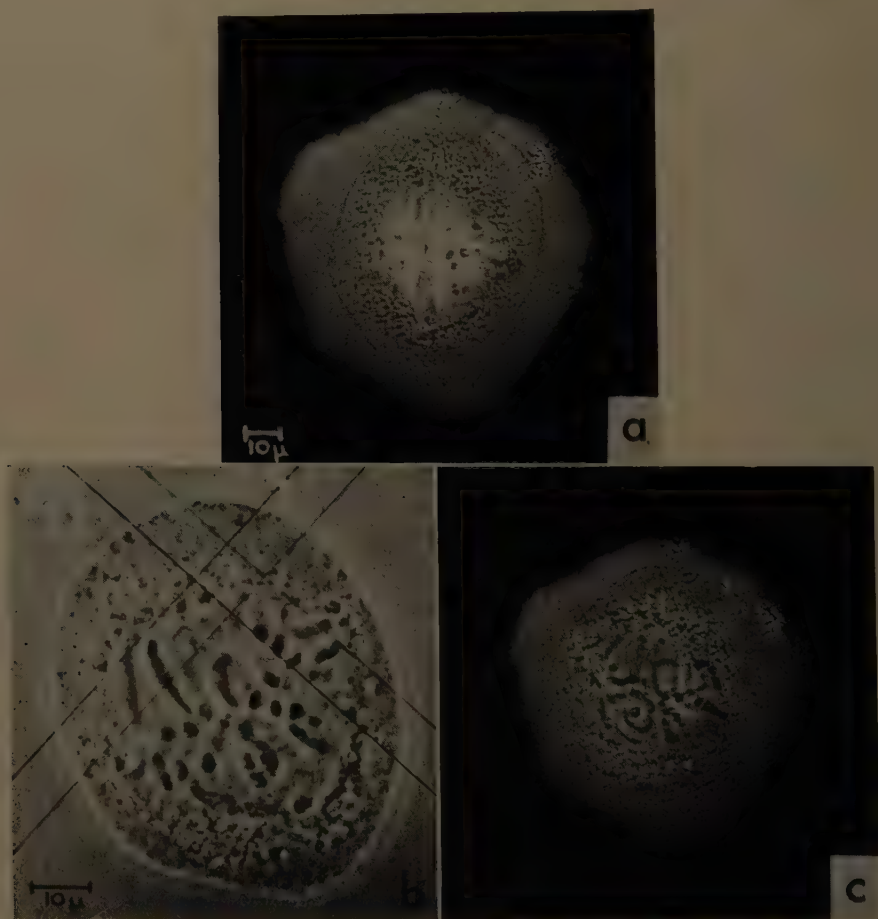


FIGURE 1. Disappearance of spindle in a metaphase cell of *Haemanthus* endosperm after heterochromatic ultraviolet irradiation of cytoplasm. Spindle axis from top to bottom. (a) By polarization microscopy, 7 min. before irradiation. Compensator set to show spindle bright. (b) By phase microscopy, 4 min. after *a*, to show region bombarded (within crosshairs). (c) By polarization microscopy, 7 min. after irradiation. Birefringence of spindle gone.

Sequelae of Spindle Disappearance

In newt cells, disappearance of the spindle after UV irradiation of a small volume of cytoplasm is followed by a very characteristic series of events that has been described in these *Annals*¹ and elsewhere.² The first event is a com-

plete loss of the metaphase chromosome configuration (deranged metaphase). This is followed by a new and orderly arrangement, that is, the metaphase chromosomes form a rosette, with their kinetochores adjacent to a common attractive region of some sort. If the amount of irradiation has been suitably

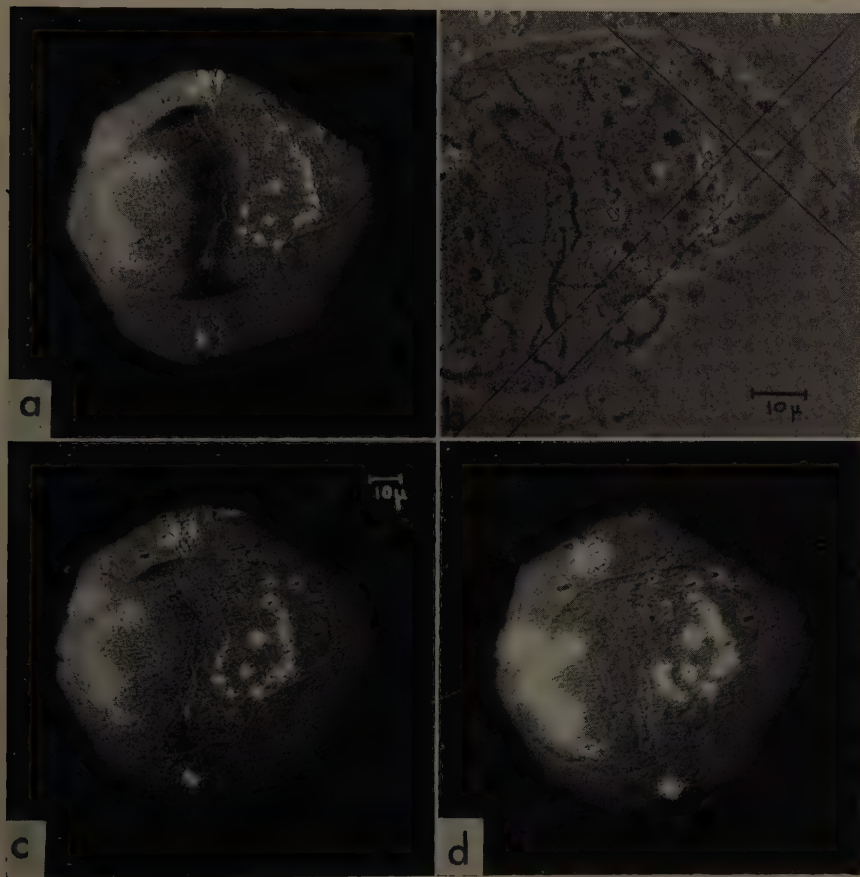


FIGURE 2. Disappearance of phragmoplast in *Haemanthus* endosperm cell after ultra-violet irradiation of cytoplasm. (a, c, d) Polarization. (b) Phase microscopy. (a) Five min. before irradiation. Compensator adjusted to show phragmoplast black. (b) Three min. after a, to show site of irradiation (crosshairs). Cell plate bisecting phragmoplast appears wavy. (c) Eight min. after irradiation. Birefringence of phragmoplast nearly gone. (d) Fourteen min. after irradiation. Birefringence no longer detectable.

adjusted, this rosette separates into two daughter rosettes, consisting of undivided metaphase chromosomes. These two rosettes, after moving about 25 or 30 μ apart, reconstruct into nuclei that appear normal by phase microscopy although they must be highly aneuploid; the cell body constricts as after a normal anaphase. Thus, in the newt cell, spindle disappearance first results in a chromosome derangement, which then is followed by a new "metaphase"

arrangement and an orderly but abnormal distribution of chromosomes to two daughter cells.*

In the *Haemanthus* cells the sequelae are strikingly different, as shown in FIGURE 3 (phase microscopy). In 3a is shown the site of irradiation in the

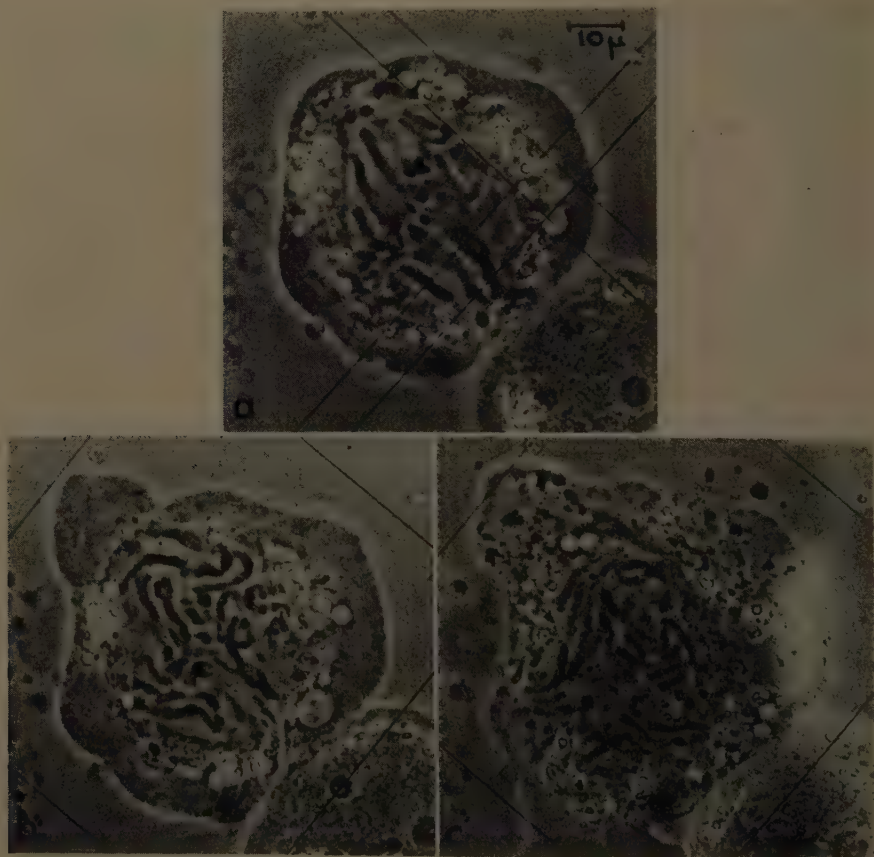


FIGURE 3. Sequelae of spindle disappearance in a *Haemanthus* endosperm cell. (a) Cell in mid-metaphase 7 min. before irradiation. Site of irradiation circumscribed by crosshairs (b) Thirty-one min. after irradiation. Metaphase configuration of chromosomes slightly deranged. (c) Six hundred and forty-one min. after irradiation. Metaphase configuration no longer recognizable.

cytoplasm of a metaphase cell. In 3b, as the spindle disappears, the chromosome configuration exhibits a slight derangement that progresses to the marked derangement shown in 3c. In some cells we have seen the chromosomes aggregate into several distinct groups, each of which became surrounded by a nu-

* Comparable observations could not be made on living sand-dollar eggs. Suitable microscopy was precluded by the small size of the chromosomes and by the abundance of granules in the cytoplasm, even in this relatively clear marine egg.

clear membrane. This distribution of chromosomes appeared quite haphazard, and in no case did we observe any orderly new mode of chromosome distribution or any division of the cell body, both of which are so characteristic of newt cells deprived of their spindles by the UV microbeam method.

It has previously been suggested^{1,2} that the orderly sequelae of spindle disappearance in the newt cells might be attributable to the centrosomes, these organelles not being seriously affected by cytoplasmic irradiation. Unfortunately, it has not been directly demonstrated that centrosomes are present at the centers of the two separating rosettes of chromosomes. However, on the basis of the centrosome hypothesis, one would predict that, in view of the lack of centrosomes in mitoses of seed plants, no orderly sequelae of spindle destruction would occur in the *Haemanthus* cells. This checks with the experimental observations.

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ASPECTS OF DEOXYRIBOSIDE METABOLISM IN RELATION TO THE MITOTIC CYCLE*

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Like many other contributors to this publication, I am interested in the question of why chromosomes duplicate at a particular time in the life of a cell. So broad an interest cannot, of course, be experimentally pursued in its entirety, and I have therefore focussed my attention on the controlled sequence of biochemical events leading to deoxyribonucleic acid (DNA) synthesis. To some students of biochemistry, studies of bacteriophage reproduction would make this area of interest appear superfluous. We already know from the work of Seymour Cohen and his associates¹ that as soon as phage DNA enters its host it induces formation of the enzyme complement necessary to its replication. There is no strong reason to suppose that a different principle of control governing DNA and enzyme induction prevails in other cells. However, for chromosome duplication in general, there remains the important distinction that in most cells its initiation is not a consequence of infection. Ultimately the stimulus to replication comes from an internal turn of events. Only at a particular point in the life of a cell does the chromosomal complement effectively induce the formation of a biochemical apparatus sufficient for its own reproduction.

Many biological factors far removed from the chromosome can and do control the duplication process. The dependence of chromosome replication and so, too, countless other subcellular events on the support of allied metabolic systems should be patent. It is idle to suppose that one agent could universally account for the occurrence or nonoccurrence of mitoses in the biological kingdom. Nevertheless there have been—and still are—many who surface-cast in familiar metabolic waters in the hope of hooking, or in illusion of having hooked, either a substance or enzyme that universally sets off mitosis.²¹ It needs to be emphasized that to reach universals through the thick diversity of cellular situations, we must not only cast deeply but also cut our frame of reference narrowly. If chromosome duplication is our objective we must ultimately address ourselves to those events that are an immediate part of the process. Conceptually, at least, we should set aside the many other extra- and intracellular conditions that in sequence or in combination make chromosome duplication possible and presuppose such conditions to be nonlimiting. In our present work we have narrowed our sights to cover only the DNA moiety of the chromosome and have circumscribed the production, energization, and polymerization of deoxyribosidic compounds as the area of investigation. What we hope eventually to learn is whether the events just listed are directly controlled by the chromosome to be replicated, or whether extrachromosomal agents regulate these events with replication spontaneously following their consummation.

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To answer such questions we have used anthers of *Lilium longiflorum* (var. Croft.). We consider the anther to be a suitable organ for study because one of its primary functions is to promote chromosome duplication in its component sporogenous tissue. As with any chosen biological material, it is by no means ideal. The anther poses as many special problems as it affords special opportunities. The problems arise largely from the fact, long noted by cytologists,^{2,3} that the layer of cells surrounding the sporogenous tissue, the tapetum, is a source of much material for the mitotic cycle. Thus reactions necessary to a particular phase of the cycle may occur outside of the mitotic cells. The complication, nevertheless, has its benefits; it permits us to discriminate between those events that must occur intracellularly and those that need not. In this way we can at least identify some biochemical steps in the replication sequence for which the immediate presence of the chromosome is not mandatory.

The first step that engages our attention is the production of precursor material for DNA. The convenience of having a distinctive set of precursors singularly directed to DNA synthesis is apparent, although some of the deoxyribosidic compounds discovered in recent years⁴⁻⁶ suggest that the direction is not as singular as we have supposed. To a small extent, at least, deoxyribosidic compounds may serve other chromosomal requirements. We shall nevertheless ignore this possible role without, in so far as we can tell, prejudicing the interpretation of our observations respecting the conditions surrounding DNA synthesis.

Even though DNA is normally confined to the nucleus, its components are not always thus restricted. Low molecular weight deoxyribosidic material has been found in the cytoplasm of unfertilized animal eggs.⁷ In such cases the material probably represents a reservoir to meet requirements for the rapid divisions that follow fertilization. The production of deoxyribosides can thus be separated in time from DNA synthesis and, to some extent, this would appear to be true in many types of tissues. Simple or conjugated deoxyribosides have been found in a variety of mammalian organs,^{8,9} in bacterial cells,¹⁰ in the roots and endosperm of corn (A. M. Bryan, unpublished), in roots and stems of beans (S. Kupila, unpublished), and in the anthers of *Lilium longiflorum*.¹¹ In the case of lily anthers we have been able to establish that deoxyribosidic compounds are not formed randomly in time, but accumulate in close temporal association with the meiotic and mitotic cycles. Each instance of accumulation is sharp and the levels of concentration high. In this respect the behavior of the sporogenous tissue is bold and unusual. Even the neighboring tapetal cells that are the seat of extensive DNA synthesis during a brief and distinctive interval in anther development manifest no such pattern. We take it for granted that the behavior of the sporogenous cells caricatures the more common situation prevailing in others at the time of chromosome duplication. However, in thus exaggerating events, it enables us to identify features of behavior that might otherwise escape notice.

Numerous events have been found to be correlated in time with the cycles of nuclear division in the microsporocytes and microspores of *Lilium longiflorum*,¹² but the most useful of these has been bud length, a relationship discovered by Erickson.¹³ Normally, the length of the developing flower bud

marks the different events in the meiotic and mitotic sequences rather closely, and it was by plotting the concentration of soluble deoxyribosidic compounds in the anther against bud length that the pattern relating these substances to nuclear division was first made apparent. It is possible, however, to show that the growth of the flower bud as a whole is not the determining factor in cycles of deoxyriboside production. In lilies that had been growing at comparatively

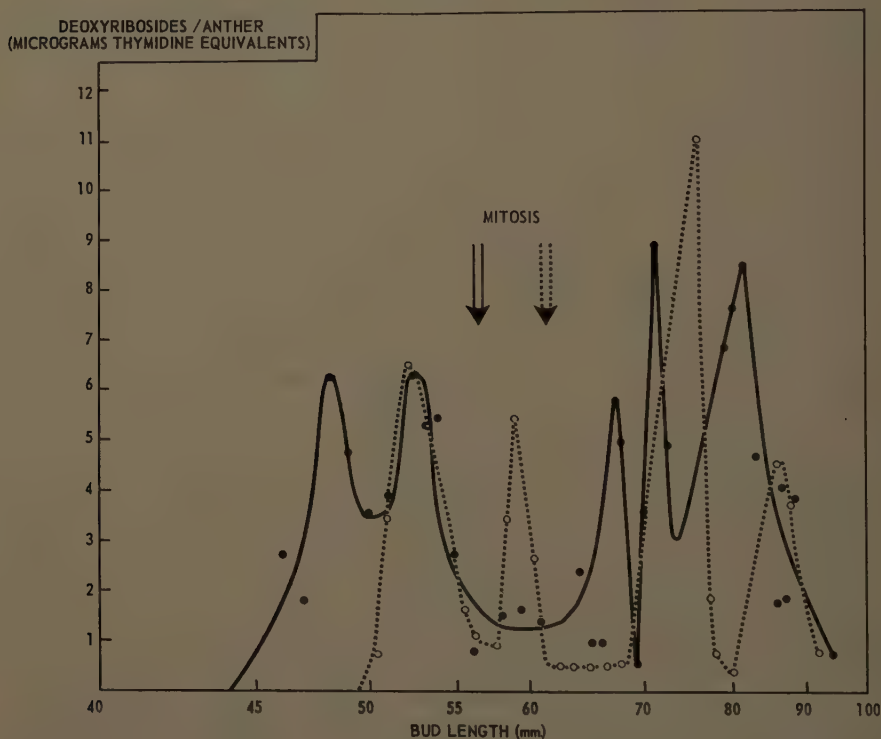


FIGURE 1. The timing of deoxyriboside production with respect to time of mitosis in anthers of *Lilium longiflorum*. The dotted curve bears on anthers in which mitosis occurred at 62 to 63 mm. bud length, and is taken from data of Foster and Stern.¹¹ The solid curve is based on data from individual buds analyzed during the summer months. The spread of mitoses during midsummer was greater than that normally encountered and this may explain the additional peak.

high temperatures (85 to 95° F.) during the summer months, microspores developed more rapidly than components contributing to bud length. The result was that microspore mitosis occurred at a bud length of 54 to 55 mm. instead of the usual 62 to 63 mm.; and, what is significant to our present considerations, the cycles of deoxyriboside production were shifted correspondingly (FIGURE 1). Although such behavior is expected, it nevertheless strengthens the impression that the anther must have supplanted the controls initially exercised by the plant on floral initiation by a set of autonomous regulatory devices. The truth of this will be apparent in some of the *in vitro*

experiments to be considered later. Perhaps the comparative immunity of anthers to growth factors administered to the intact plant¹⁴ is due to this autonomy of control.

The degree of precision in the timing of deoxyriboside production and consumption is the point we wish most to emphasize. In testing individual anthers, the span of bud length over which a particular peak in deoxyriboside concentration occurs is no greater than 3 mm. If large groups of pooled anthers are analyzed the span of the peak may be reduced to 1 or 2 mm.¹¹ Three millimeters of bud length represents approximately 12 hours of growth but the actual duration of the deoxyribosidic pool is probably less if we allow for the imperfect fit between bud length and microspore development.¹³ The significance of a 12-hour span may be best appreciated from the fact that the time from microspore birth as a product of meiotic divisions until completion of post-mitotic DNA synthesis is 20 to 25 days. Measured against the scale of the cell's own life span deoxyriboside production and consumption are brief events. The sharpness of the situation is given additional emphasis by the close but separate occurrence of two cycles of deoxyriboside production and consumption at each brief interval of DNA synthesis.¹¹ The reason for this we do not yet know; it may well reflect distinct intervals of DNA synthesis such as reported by J. H. Taylor in this monograph and as found by Lima de Faria¹⁵ with respect to heterochromatin and euchromatin, or it may mean that the second pool of deoxyribosides is not incorporated into DNA. Whichever it is, we may draw the significant conclusion that the mechanism for deoxyriboside production has a periodicity as precise as chromosome reproduction itself. The regulation of DNA replication thus has its counterpart in the regulation of precursor formation.

In the experiments just described the nature of the deoxyribosidic material appearing in pulses has not been specified. Although we have by no means fully analyzed the various pools, we have sufficient information to provide a pertinent picture. If one chromatographs extracts of anthers harvested at an appropriate bud length with butanol-water as the differentiating solvent, the major portion of the deoxyribosidic pool distributes itself in a pattern corresponding to the deoxyribosides of DNA¹² (FIGURE 2). We take this to mean that in the samples tested, phosphorylated derivatives are comparatively scarce since such compounds do not migrate in a butanol-water system. That the mobile components are largely deoxyribosides is indicated by the correspondence in their R_f values when individual spots eluted from the butanol-water chromatogram are rerun with isobutyric NH_4OH solvent. Thus far, by a combination of analytical methods we have positively identified only one component, deoxyguanosine. There is little doubt, however, that the purine/pyrimidine ratio in the pool is approximately 1, since incubation for 2 hours in 0.1 N HCl at 37° C. destroys one half of the deoxyriboside linkages. Some forms of conjugated deoxyribosides may also exist in the pool. Initially we presumed a large fraction of the pool to be thus constituted because of our finding that a substantial increase in the amount of microbiologically detectable deoxyribosides occurred after treating methanolic extracts of pooled samples with commercial phosphatase.¹¹ However, efforts to repeat these results on individual

buds freshly harvested have indicated no more than 10 to 15 per cent to be thus bound. We cannot explain the discrepancy except for noting the instability of much of the deoxyribosidic material in storage. The picture as a

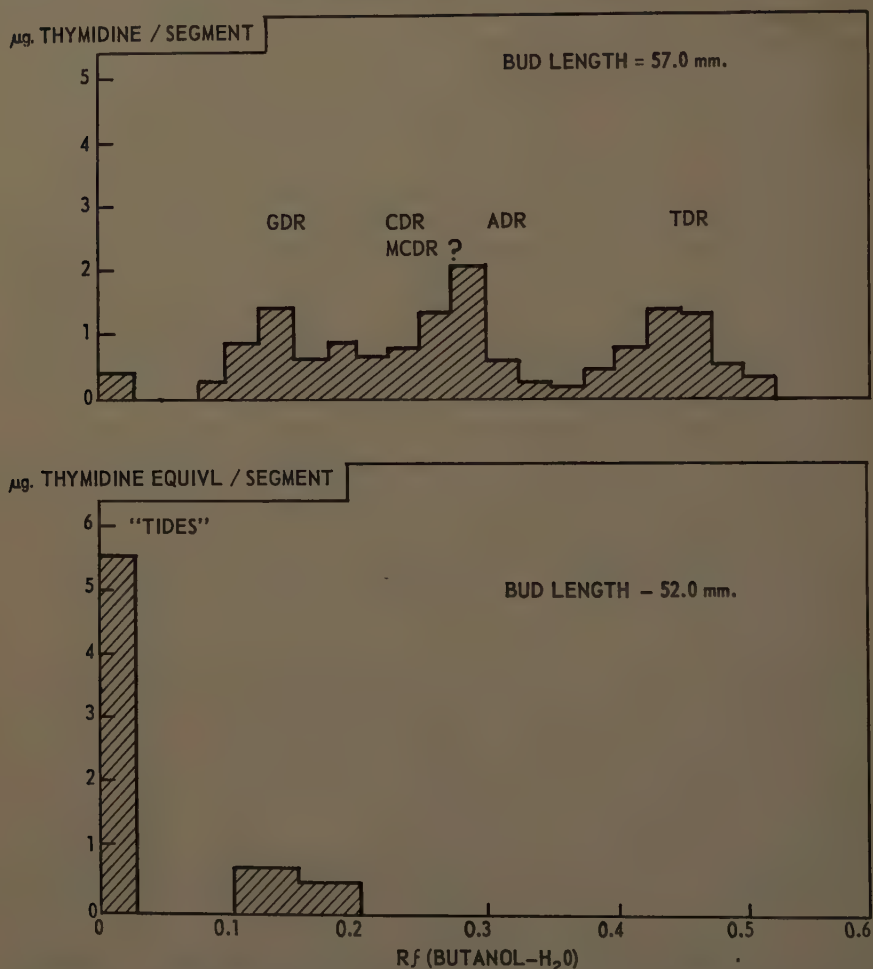


FIGURE 2. Comparison of deoxyriboside pools in anthers of two different buds. Both were extracted with 70 per cent methanol and a portion of the extracts developed on paper with butanol water. Broad strips equivalent to an R_f of 0.05 were cut and their deoxyriboside contents determined.

whole, however, is amply clear; the ratio of nonphosphorylated to phosphorylated derivatives is on the average about 9:1.

The above analytical evidence poses a simple question: If phosphorylation is a necessary prelude to polymerization, how can the pool of precursors appearing so close in time to polymerization be of the nonphosphorylated variety? There are two possible answers to the question if one makes the assumption

that phosphorylation is necessary. Either there is a continuous and steady phosphorylation of deoxyribosides with immediate incorporation of the product into the polynucleotide form, or the interval of phosphorylation, although distinct, is very brief compared to that of deoxyriboside accumulation. The observed composition of the pool is consistent with either alternative since such composition represents the average of a large number of anthers. However, the issue may be satisfactorily resolved by testing individual ones. If a brief but distinct interval of phosphorylation follows deoxyriboside production, then extracts of occasional anthers should show a major proportion of the phosphorylated derivatives. This is precisely what we have found (FIGURE 2). It therefore appears that the first two steps of the sequence in DNA replication have a measure of autonomy with respect to one another and a high degree of temporal regulation. The postulated regulatory process must thus account not only for the qualitative complement of enzymatic activity induced within a rather brief time interval but also for a sequential induction within the bounds of that same interval.

TABLE 1
DISTRIBUTION OF DEOXYRIBOSIDES AND DNA IN LILY ANTHERS

Bud length (mm.)	Thymidine equiv. (μ g.)		DNA-P (μ g.)	
	Wall	Microspore core	Wall	Microspore core
50.5	4.5	4.0	6.5	0.6
51.0	0	0	8.0	0.8
53.0	3.9	4.9	5.8	0.6
54	4.4	3.6	4.3	0.3
58.5	3.8	3.8	3.8	0.3

Values given are per anther. For nature of "core" material see text.

One point bearing strongly on the control of the deoxyriboside-producing mechanism is the site of its action. It might be supposed that a precursor most intimately bound in time and function with DNA replication would be produced in the vicinity of the chromosome. This is not necessarily the case. Y. Hotta has devised in our laboratory a simple technique for removing the core of microsporal tissue from the locule of each anther without the use of liquid or mechanical pressure on the adjacent portions of the anther (details of the method will be described elsewhere). In this way diffusion of soluble material is avoided and cellular injury reduced to a minimum. The core thus removed contains 80 to 90 per cent of the microspores originally present in the anther and a corresponding proportion of the substrate in which the microspores are embedded. A variable amount of tapetal tissue contaminates the core but this fact is irrelevant to the particular point at issue. If the distribution of deoxyribosides is determined in anthers at or close to their peak concentration, it is found that approximately half of the pool is located outside the microsporal core (TABLE 1). The result does not permit us to say what proportion, if any, of the deoxyribosides is present in the microspores but it

does permit us to conclude that at least half is present outside of them. In a few instances we washed the microspores with sucrose solution to free them of adjacent material and in these cases none of the deoxyribosides present in the anther were found in the microspores. Although we cannot eliminate the possibility of soluble compounds being washed out of the cells, the result is at least consistent with the view that initially all the deoxyribosides are present outside the microspores in the adjacent regions of the anther. The fact that a major portion of the deoxyribosidic pool destined to be utilized by the microspores is outside of them leads to the conclusion that the deoxyribosides originated there. Thus the control associated with one of the initial steps in chromosome replication clearly extends beyond the cells directly involved.

One might at first suppose that the regulatory source also lies outside the microspores. If this were the case it would by no means simplify our problem since we would still need to integrate the activities of intra- and extracellular regulators. From the developmental picture, however, the presence of a timing device immediately outside the microspores appears to be the less likely possibility. The adjacent tapetal tissue in which the deoxyribosides may originate begins to degenerate some time before mitosis. Since this degeneration does not have the sharp periodicity characteristic of deoxyriboside production, and since the failing organization of the tapetum makes it an unlikely source of regulation, we suppose that the timing originates in the microspores. If so, it is important to note that the event timed has no immediate association with the chromosomes. The total separation of deoxyriboside production from chromosome replication is of course quite evident in the mutant strain of *Lactobacillus* that depends entirely on an exogenous supply of deoxyribosides for growth.¹⁶ However, unlike the bacterium, the microspore cannot have lost a genetic capacity evident in somatic tissues to synthesize deoxyribosides intracellularly. Thus, within the same organism, different enzymes may be activated in different cells to meet the needs for DNA synthesis. Furthermore, if such activation arises from gene-controlled enzyme induction, then clearly, unlike the situation in phage replication, the same group of genes is not consistently involved in the replication process.

A distinction thus needs to be drawn between the agent of regulation and the mechanism regulated even in so highly coordinated a set of events as that embracing deoxyriboside production and DNA synthesis. To provide concrete evidence for the wisdom of this distinction is, however, a difficult matter. It is possible that the circumstances under which deoxyribosides are produced in connection with microspore mitosis may provide a pointer in the desired direction.

Chromosome replication in the sporogenous tissue occurs under two different sets of conditions. At premeiosis the anther is steadily increasing in size and there is a parallel increase in total DNA. At mitosis, on the other hand, growth in size is near completion and the total DNA, having reached its maximum earlier in development, is now falling¹¹ (FIGURE 3). This fall is largely attributable to the breakdown of tapetal tissue.¹² Presumably, meiotic DNA synthesis depends upon a *de novo* production of deoxyribosidic compounds, although this point has been questioned.^{3,17,18} During the mitotic interval it appears

probable, at least on superficial grounds, that the periodic pools of deoxyribosides originate from the degradation of DNA. If so, two phenomena must be accounted for: the magnitude of DNA breakdown that far exceeds the amounts of deoxyribosides accumulated; and the sharp periodicity of such accumulations against a background of gradual DNA breakdown.¹²

The critical issue in the phenomenon is to demonstrate that the deoxyribosidic pool arises from preexisting DNA and that this same pool is used for DNA synthesis. The best method of approaching the issue is by labeling nonmicro-

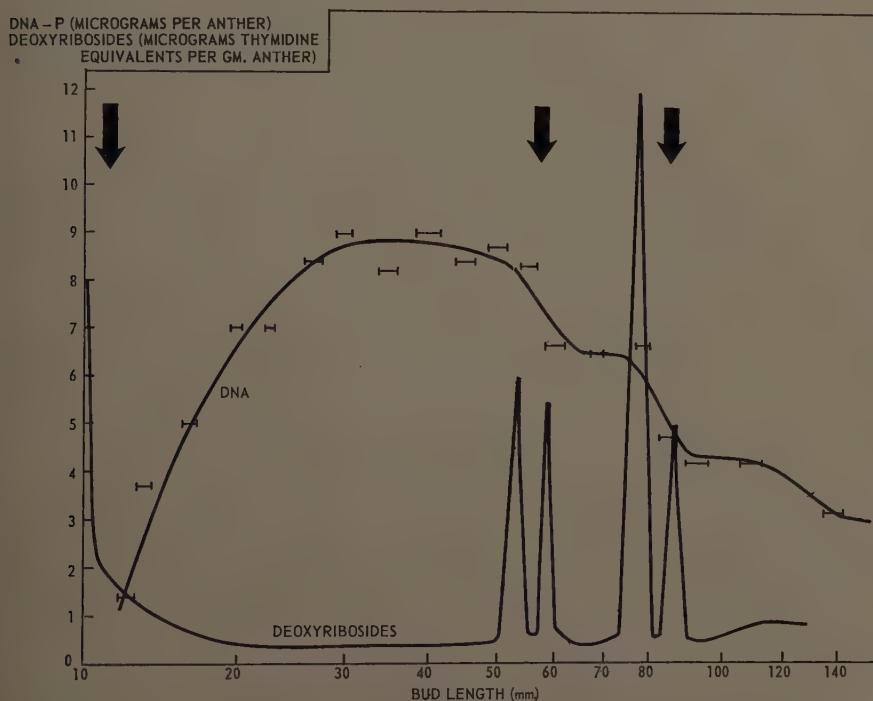


FIGURE 3. The accumulation of deoxyribosides in relation to changes in total DNA content of developing anthers. Curve for DNA represents the sum of DNA extracted first by cold 0.25 M NaCl and then by hot 10 per cent NaCl (Stern¹²). Vertical arrows indicate intervals of DNA synthesis in microsporocytes and microspores.

sporal DNA during early stages of anther development and following the label through the pool into the newly synthesized DNA. Takats¹⁷ has made good progress in that direction using radioautographic techniques; his findings, when completed, may largely settle the question. However, for the kind of studies here undertaken, labeling of extra-microsporal DNA poses enough technical barriers to discourage its use. We have therefore turned to two other approaches. Our first aim was to see whether an equation could be drawn between the disappearance of a distinguishable fraction of DNA and the formation of the deoxyriboside pool. Since the major portion of the DNA lost from the anther was not accumulated as deoxyriboside, it appeared possible that a

particular reservoir of DNA amounting to about 10 per cent of the total was involved in the reaction. To date, results have been equivocal even though the DNA of the anther can be subdivided into two classes according to solubility.¹² Thus we have no direct evidence for the origin of the deoxyriboside pool. Our second but indirect approach was to look for enzymatic systems that should account for the transformation. In this we have been successful although our studies are still incomplete.

To put the substance of our findings ahead of relevant details, it may be said that anthers contain a set of enzymes capable of degrading polymerized DNA into its deoxyribosidic constituents and that one component of the system appears to be periodically rather than continuously active. The presence of the enzyme system was demonstrated in tissue homogenates. In these we found that deoxyriboside formation kept a close pace with over-all DNA degradation. Thus, if polymerized calf thymus DNA was incubated for 2 to 3 hours with anther homogenate, and the products resolved by paper chromatography, these turned out to be largely deoxyribosides. If, alternatively, incubations were carried out for 10 to 20 min., and the acid-soluble products assayed microbiologically, little additional stimulus to growth occurred on pretreating the products with snake venom phosphatases. This was in marked contrast to the products of crystalline deoxyribonuclease activity, which had a comparatively low growth prompting activity prior to phosphatase treatment. The enzymatic properties of anther homogenates are therefore consistent with the virtual absence of polydeoxynucleotides and the predominant presence of deoxyribosides in anthers developing *in situ*.

Two enzymes of the system, phosphomonesterase and phosphodiesterase, show no marked periodicity in activity. The first of these can be demonstrated at most stages in anther development by incubating homogenates with any of the four principal deoxynucleotides or by placing in a drop of deoxynucleotide solution an anther that has been cut close to the base to facilitate diffusion. Under both conditions deoxyribotides are dephosphorylated to deoxyribosides and it is this reaction that probably accounts for the preponderance of deoxyribosides in the pools we have analyzed.

The presence of a phosphodiesterase that would cleave the primary products of DNAase action is most conveniently demonstrated by the use of an artificial substrate, calcium bis *p*-nitrophenyl phosphate. On a dry weight basis, 2 per cent of which is protein, the anther is 5 to 10 times as active as snake venom, a principal source of the enzyme. Comparatively, therefore, diesterase activity is exceedingly high. Unlike snake venom, the enzyme is most active in the acid range (TABLE 2); at most bud lengths, the peak of activity is at a *pH* of 4.7. There is some activity on the alkaline side of neutrality but at 7.8 this becomes negligible. A number of buffers—phosphate, Tris, and veronal—have proved to be partly or completely inhibitory.

DNAase activity has been measured in terms of acid-soluble products formed. Optimal activity is in the acid range (TABLE 2); Mg ion does not stimulate; 0.2 *M* phosphate buffer is inhibitory, and no activity is observed in Tris buffer at *pH* 7.3. If anthers are homogenized in 0.35 *M* sucrose and the homogenate centrifuged to yield a clear supernate, the latter fraction, depending upon

bud length, contains about 20 to 70 per cent of the activity with an optimum around 5.0 whereas the sediment that accounts for the remainder has an optimum in the vicinity of 6.5. At bud lengths thus far tested, the major portion of diesterase activity has always been found in the supernatant fraction. We have yet to determine the extent to which the two DNAase fractions vary in the course of development.

The site of DNAase action appears to be much the same as that of deoxyriboside formation. There is a variable distribution in activity between microsporal core and remainder of the anther, the former at times accounting for 60 per cent of the total. The significance of the distribution may be better appreciated if we consider another enzyme that catalyzes the conversion of deoxycytidine to deoxyuridine. Homogenates of anthers or intact anthers cut at the base have a high capacity for such conversion. Within one hour at

TABLE 2
CHARACTERISTICS (pH) OF DNA BREAKDOWN SYSTEM IN ANTHERS OF *LILIU*M

pH	DNAase		Diesterase	
	Homogenate	Sediment	Sediment	Supernate
4.1	13	4	0.04	0.42
4.7	28	7	0.09	1.11
5.0	34	12	0.122	0.9
5.25	35	17	0.130	0.72
5.7	38	32	0.105	0.72
6.1	35	28	0.110	0.65
6.6	34	32	0.120	0.65
6.6 (PO_4)	0	0	0	
7.3 (Tris)	0	0	0	

Values for DNAase are micrograms of DNA-P rendered acid-soluble by an anther in 1 hour at 25° C. Diesterase activities are presented as optical density changes at 440 $m\mu$ in 20 min. at 25° C. In each diesterase assay 0.03 of an anther was used, and the total volume of the incubation mixture after dilution for reading was 5.0 ml. For other conditions see text.

30° C. an anther can convert some 200 times its normal peak content of deoxycytidine. Nevertheless, in so far as we can tell, deoxyuridine cannot be converted to thymidine, which is the only logical route if it is to serve as substrate for DNA synthesis. Hotta has been able to demonstrate the conversion of deoxycytidylic to thymidylic acid. Under the conditions of the experiment deoxycytidine may be used, but its conversion occurs only after phosphorylation. We have yet to find appropriate conditions for deoxyuridine phosphorylation in the interval surrounding microspore mitosis if the reaction is at all possible in the anther. There is thus an enzyme that can effectively catalyze the removal of a necessary constituent from the deoxyriboside pool and thereby control DNA synthesis. This enzyme does not do so, however. Deoxycytidine is present in the pool, and this is probably the case because, unlike the DNAase system, the enzyme is largely confined to the wall cells of the anther. The contrast in localization of deoxycytidine deaminase and DNAase respectively adds to the circumstantial evidence that the

latter enzyme may have some direct role in the deoxyriboside metabolism of the microspores. There is, moreover, one additional point worth mentioning.

If the microspores are surrounded, as they appear to be, by a curtain of DNAase it seems unlikely that any large pieces of DNA would reach the microspores from adjacent tissues, a possibility occasionally given credence.¹⁸

If DNAase activity alone is the limiting factor in deoxyriboside production, it should manifest a periodicity coincident with it. In this respect the evidence is incomplete and must remain so until we have at least followed the behavior of each of the two DNAase systems through anther development. However, the information we have thus far obtained from analyzing single or groups of anthers at different bud lengths leaves no doubt that a periodicity does exist in total DNAase activity (TABLE 3). In premitotic interphase DNAase does not appear until close to the time when deoxyribosides accumulate, nor does

TABLE 3
VARIATIONS IN DNA BREAKDOWN DURING ANTHER DEVELOPMENT

Bud length (mm.)	Activity	Bud length (mm.)	Activity	Bud length (mm.)	Activity
42	0	51 (2)	22	87	27
44	0	52	18	92-97 (5)	25
45 (2)	0	60	15	99	20
46.5	0	64	10	104	28
46.8	24	62-64 (4)	0	100-106 (4)	16
47	17	68-69 (5)	0	109	0
47	1.8	71	0	110	0
49	0	76	20	112 (2)	0
50	29	75-79 (4)	7	114-126 (6)	0
		82-87 (4)	31		

Activities calculated as micrograms DNA-P rendered acid-soluble per anther at 25° C. for one hour. Incubation system contained .15 ml. anther tissue 0.075 ml. of 0.5 *M* acetate buffer (pH 5.25) and 0.5 ml. calf thymus DNA (1 mg./ml.). Values in parentheses represent number of buds used in pooled samples; where no such values are given, only one bud was tested.

any persist beyond 105 mm. when no further accumulations of deoxyribosides have been noted. Between these points it is evident from TABLE 3 that depending upon bud length the enzyme may or may not be present.

Should it prove that deoxyriboside pools do arise from preexisting DNA, it is nevertheless doubtful, despite the fluctuations in DNAase activity, that this enzyme is the immediate source of regulation. We may conceive of its role as a macroregulator in the sense that its presence over a given interval of time is necessary to the more finely resolved fluctuations occurring during that same interval. The grounds for believing this are as follows: although we have not yet found instances when deoxyriboside production occurs in the absence of DNAase, we have found a few instances when DNAase is present and deoxyribosides absent. If anthers excised from buds measuring 45 to 46 mm. are cultured in phosphate (but not acetate) buffer at 15° C. DNAase appears after 3 to 5 days suggesting that the regulatory mechanism is unimpeded. By contrast, cultured anthers that have an initially high DNAase activity may retain this for an equally long period of time despite the fact that under the same con-

ditions deoxyriboside production remains cyclical. In FIGURE 4 we have plotted the behavior of deoxyribosides in anthers cultured for a period of approximately 18 hours at 15° C. It is evident from these experiments that aside from those anthers that do not undergo any change in deoxyriboside content, an increase is noted only in those that have an initially low level and a decrease only in those that have an initially high level. *In vitro* and, at least to some

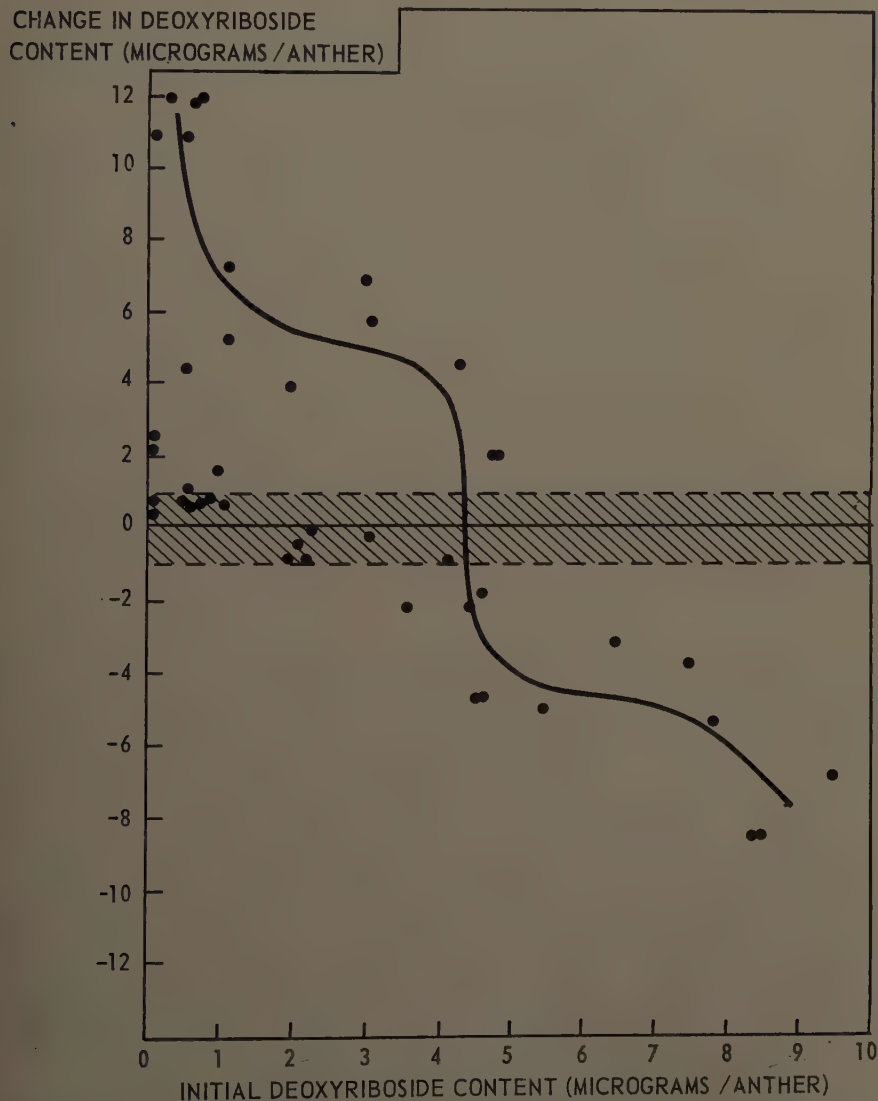


FIGURE 4. Changes in deoxyriboside content of anthers cultured at 15° C. for approximately 18 hours in Taylor's medium or phosphate buffer (6.5). Filaments of anthers were immersed in 0.1 to 0.2 ml. of solution.

extent, *in vivo* DNAase persists over much longer intervals than are required for the formation and disappearance of deoxyriboside pools. Thus if the deoxyriboside pool arises through DNAase activity, the periodic fluctuations in the pool may well be controlled by something other than the enzyme itself. Substrate, as such, cannot be a limiting factor since it is present in excess over an extended period of time. Whether the accessibility of enzyme to substrate, or whether the induction of a specific type of DNAase activity is the limiting agent can be resolved only by further study.

In conclusion, we recapitulate our views and interpretations. We regard the anther as an organ evolved to facilitate, among other things, nuclear divisions in the germ line. We have set aside in our studies the many accessory agents that can limit chromosome replication at developmental points outside the immediate bounds of the replication process itself. We have drawn a starting line at the point where deoxyribosidic material is produced because, on the whole, the principal fate of such material is to serve as substrate for DNA synthesis. In terms of the life span of the cells in question, deoxyriboside production is a sharply demarcated event, brief in duration, and precisely timed with respect to chromosome reproduction. It occurs extracellularly, and the nonphosphorylated end products probably penetrate readily into the microspores. The latter appear to be surrounded at appropriate times with an effective DNAase system such that polymerized DNA or large fragments of it would be degraded in passage. This same system may be responsible for deoxyriboside production. The phosphorylation that follows deoxyriboside production is segregated in time from it, but appears to have the same sharp characteristics. Hotta's initial studies indicate that phosphorylation occurs in the microspores but we do not yet know whether it occurs there exclusively. It would appear, moreover, that the first step in phosphorylation, the conversion of deoxyriboside to deoxyribotide occurs as a single event without concomitant pyrophosphorylation.

These results are much too limited in scope to permit one to make more than a few suggestive points about the regulatory scheme embracing the production of deoxyribosides and their eventual polymerization. In considering the periodicity of events a distinction should be drawn between their mode of regulation and the agent of their regulation. By "mode" we mean the enzymes and substrates comprising each event; by "agent" we mean those factors that induce the formation of the appropriate biochemical systems. The first of these is the more readily accessible to experimental attack since the mechanisms involved—for example substrate limitation, energy limitation, presence or absence of enzyme—are comparatively familiar. Many instances of mitotic regulation involving one of more of the above mechanisms have been described, although often such descriptions have been gratuitously given a stamp of finality.²¹ Leaving aside the metaphysical question of ultimate cause with respect to the periodic changes in metabolic pattern during the mitotic cycle, there is a need to identify the immediate agency that brings them about.

Two points that have a bearing on the above considerations have emerged from our studies of chromosome duplication in microspores. A comparison of these with somatic cells of the lily suggests that DNA synthesis may be brought about by different sets of enzymatic mechanisms among cells of the

same genome. If this is true, then more than one agent of regulation probably governs chromosome duplication, the effectiveness of a particular agent being dependent upon specific cellular conditions. Furthermore, the apparent pattern of the metabolic chain from deoxyribosides to DNA as a sequence of discrete enzymatic activities rather than as a tandem arrangement of catalysts over which substrate continuously moves, points to a sequential induction of enzymatic activities by more than one regulatory agent. We do not conclude that such induction is a universal mode of regulation, but we do conclude that simultaneous induction characteristic of phage is not. We have yet to determine whether the sharp temporal separations of enzymatic activities have their basis in a correspondingly sharp separation in times of enzyme formation. Were this the case, it would be fashionable to assume that the phenomenon studied covers a sequence of induced syntheses of enzymes. This point we hope to clarify in the future.

If we suppose that induced enzyme syntheses are at the root of the various pulses in metabolic activity we are led, not by logical necessity but by current intellectual persuasion, to consider the gene as the agent in question. If so, our results invite two comments. First, it is unlikely that the genes constituting the agent are activated as a block since the evidence points to more than one species of enzymes catalyzing a particular phase of DNA synthesis. This interpretation is consistent with, if not supported by, the sequential ordering of enzymatic activities. Second, if deoxyriboside production is dependent upon a gene-induced enzyme, then, from the evolutionary standpoint, survival value would be much enhanced if the chromosomal complement were equipped with several genes, each capable of somehow inducing deoxyriboside formation. From this it is not difficult to see that even within a particular species of cell more than one mechanism for deoxyriboside production may be available, if not active, during the interval of DNA synthesis, although multiple routes of synthesis cannot be excluded.

To stamp the gene as regulatory agent does not, however, as much as provide a framework for solving the problem of why a chromosome duplicates at a particular time. Nor is it any more fruitful to look for a gene-activating substance since we would only extend the agony of our searches along an infinite chain of cause-effect relations. Such substances may well exist, but whether in abundance or scarcity, we must shift our frame of reference to the developmental one in order to provide a logical basis for solution. Time is an inviolable parameter of life and its cumulative effects on a cell, whether classified as development, maturation, or aging, is the ultimate causal source of the regulation of chromosome duplication. That such effects may be altered to a variable degree by environmental conditions, by pathogenic or chemical agents, is beside the point. That the duration of its action may be brief or extended is a secondary consideration. What counts in our primary resolution of the problem is the possibility of designating a system of change in which time is the effective mover. Specifically, we must determine whether the chromosome itself undergoes any cumulative change so as to activate certain genes or to make it susceptible to replication. Moreover, if the chromosome is not thus involved, then we must look for the locus of induction in extra-chromosomal regions. In this frame of reference no single activating substance is manda-

tory, and a progressive shift in steady states of one or more metabolic systems, possible. Indeed, it looks as though the term "regulatory substances" offers more comfort than explanation. Recent attempts to set up an interplay of degradative and synthetic enzymes as a mechanism of tilting the balance in favor of or against replication¹⁹⁻²⁰ are far more likely to represent efforts at description of consequences rather than analysis of causes in cells poised for reproduction. The behavior of the anther marks too strongly the close relationship between periodic induction of enzymatic activities and periodic replication to make room for an extended tug of war between enzymatic systems of opposing direction. In the last analysis, it would appear that in cells in which chromosome replication occurs without the intervention of an external stimulus, the heart of the phenomenon lies in a process mediated by the cumulative action of time and occurring in cytoplasm or nucleus, or both.

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THE ANALYSIS OF CELL REPRODUCTION*

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In an earlier monograph entitled *The Mechanisms of Cell Division* the late E. G. Conklin (1950) made a remark to the effect that the progress of this subject was comparable to the locomotion of a squid, which moves backwards emitting quantities of ink. That may have been true of the study of cell division in the late 1940s, but it is not true today. Unfortunately, one cannot be as witty about success as about failure, as every philosopher who has been unfunny about the nature of humor has demonstrated.

This monograph, while not nearly a complete summary of recent progress, will nevertheless provide convincing documentation for the statement that, while we do not understand cell division (whatever it means to "understand" so rich a constellation of amazing processes), we are in a period of deepening insight as well as of proliferating information.

Ten years ago a great era of observational cytology had been completed. Good eyes and gifted imaginations had extracted a remarkably rich body of knowledge and inference, unanswerable questions had been formulated and summarized (for example by Schrader [1944]), and it was clear that newer and finer methods were now in demand. Beginnings had been made toward an experimental analysis of cell division, and the earlier Academy monograph is a revealing record of those pioneering sallies. The progress that we are now recording is a measure of the contemporary renaissance of cytology, which apparently includes even a rejection of the old name for a fresh and uncommitted name, cell biology.

In this paper, I intend to outline the elements of the present-day *analysis* of cell division. I use the term analysis in a quite literal sense; we can begin to study something as complex as cell division only by dealing with unit processes, one by one, and with their interactions, two by two. We can interpret complexity quite literally if we can discover distinguishable and, ideally, experimentally dissociable processes. "Complex" does not necessarily mean "hard to understand." It should mean, simply, "composed of many parts and of definite relationships between the parts." If we are taking a sanguine view of our progress toward an understanding of cell reproduction, it is because we have been finding ways of identifying the unit processes, of studying those immediately accessible to our methods and formulating reasonable questions about others that will become answerable as our experimental powers increase. Let us now justify these optimistic estimates, considering briefly our present image of a number of the processes into which the cell's scheme of reproduction have been dissected.

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Reproductive Events of Cell Division

One of the great advances in insight has come from the separation of the genuine reproductive processes of the cell cycle and those processes by which the products of reproduction are distributed between daughter cells. Basically, there are only two truly reproductive processes, defined as the generation, by so-called self-reproducing units, of copies of themselves. The first is the reproduction of the chromosome substance, for which the ideal "tracer" is the doubling of DNA. We need not even commit ourselves as to the genetic role of DNA alone or conjugated to protein. Even if the evidence for the current equation, $\text{DNA} = \text{genes}$, were not almost overwhelming, DNA in its traditional role as "chromatin" would still be a reliable signal of chromosome substance. The second reproductive process is the doubling of the mitotic centers, seen in animal cells as the duplication of the centrioles. The debate concerning the presence of centers in plant cells has not been very active in recent years. Certainly, centrioles as such have not been seen in plant cells with the light microscope or with the electron microscope. If they existed in the form observed in animal cells by the electron microscopists—a cylinder about 2000 Å long composed of a ring of about nine sets of rodlets or tubules—they should be seen in the micrographs that illustrate the paper of K. R. Porter, but they are not seen. Nevertheless the behavior of chromosomes in plants has led many cytologists to assume the existence of organizing centers or "spindle organizing bodies" (Schrader, 1953). One is inclined to conclude that if plant cells do not have centrioles they do possess equivalents that we may call whatever we please. Out of deference to those who are impressed by the absence of morphologically distinct centrioles, let us call the polar organizers in plant mitosis "euphemisms."

Thanks to the advances of cytochemistry, especially of spectrophotometry and of autoradiography, we may consider it proved that the doubling of chromosomal DNA and of its associated histones takes place before division. In this monograph some of the cytochemical evidence on this and related problems of chromosome reproduction is summarized by J. H. Taylor. H. Stern gives us a beautiful example of a biochemical analysis of the same problem, tracing the timing of DNA synthesis back to the precursor level in a synchronized cell population.

We can go beyond the statement that these reproductive syntheses take place normally during interphase or at least before the stage of mitosis at which chromosomes are in movement. We can also say that the condensed mitotic chromosome is *incapable* of DNA synthesis. This follows from Taylor's experiments with colchine and from other experiments (Bucher and Mazia, 1960) in which the synthetic capability of chromosomes blocked in their condensed condition was studied. There is no incorporation of labeled thymidine into the condensed chromosomes even though it has been shown that the agents used to block them in mid-mitosis, colchicine and mercaptoethanol, have no effect on DNA synthesis during interphase. In so far as we can now tell, the genetic material of chromosomes is doing nothing during division; it is coming along for the ride.

We have recently studied the reproduction of the centers in the sea urchin

egg in some detail (Mazia *et al.*, 1960). Since this work has been published, I need summarize only the conclusions: (1) the centers are normally duplex structures, and their reproduction is a process of making four units from two; (2) the true biosynthetic reproductive event, which I may call the "conception" of a new centriolar unit, is experimentally dissociable from the later events that complete the reproductive cycle of the centriole: the development of the progeny to the point where they can function autonomously to form a pole and the splitting or "parturition" of the products of the duplication; and (3) although the conception of new potential poles is experimentally separable from the parturition of the reproductive products, both take place at about the same time in the mitotic cycle, during the anaphase-telophase period. This makes the reproduction of the centers the earliest process assignable to a given mitosis, taking place before the reproduction of the chromosome substance.

In analyzing these findings, the interpretation of which is summarized in FIGURE 1A, we are called upon to examine the nature of the reproduction of large and complex assemblies such as chromosomes and centers a little more closely. In doing so, let us distinguish between what I call the *fission* model of a reproductive process and the *generative* model. In the fission model, of which a prime example is the Watson-Crick theory of the duplication of the DNA molecule, each part of the parent unit reproduces itself and perhaps gives one half of its substance to each daughter. The products are described as sisters, not as parent and daughter. Biosynthetic duplication and the splitting-apart of the products are essentially simultaneous processes. In the generative model, only part of the structure is self-reproducing in a literal molecular sense. Its reproduction gives rise to a germ or seed that determines the development of the daughter unit without further dependence on the parent unit. Thus conception, development, and parturition will be distinguishable successive stages whereby a daughter, equivalent to the parent unit, is produced. The reproduction of all familiar complex units follows a generative pattern. This has long been obvious in the case of the reproduction of elephants, but has only in recent years become equally certain, in a slightly different sense, for the reproduction of a bacteriophage (in the bacteriophage, the parental soma is sacrificed before the conception of progeny; in the elephant, after the period of reproduction).

One of the characteristics of functional systems reproducing by a generative mechanism is that at least two generations must exist at all times; the parental generation that is complete and operational and the daughter generation that is developing toward functional competence. Thus if the centers do reproduce by a generative mechanism, it is easy to see why they are double and why, if division is blocked long enough to permit the maturation of the younger generation of units, a cell may be induced to divide into four without the conception of new centers (FIGURE 1B).

It is even possible that the chromosomes reproduce by generative mechanism. This might not be evident so long as we consider only the genetic material, whose reproduction would involve some kind of a fission mechanism almost by definition. However, the chromosome consists of more than DNA and more than deoxyribonucleohistone. There are other proteins, RNA, nucleolar sub-

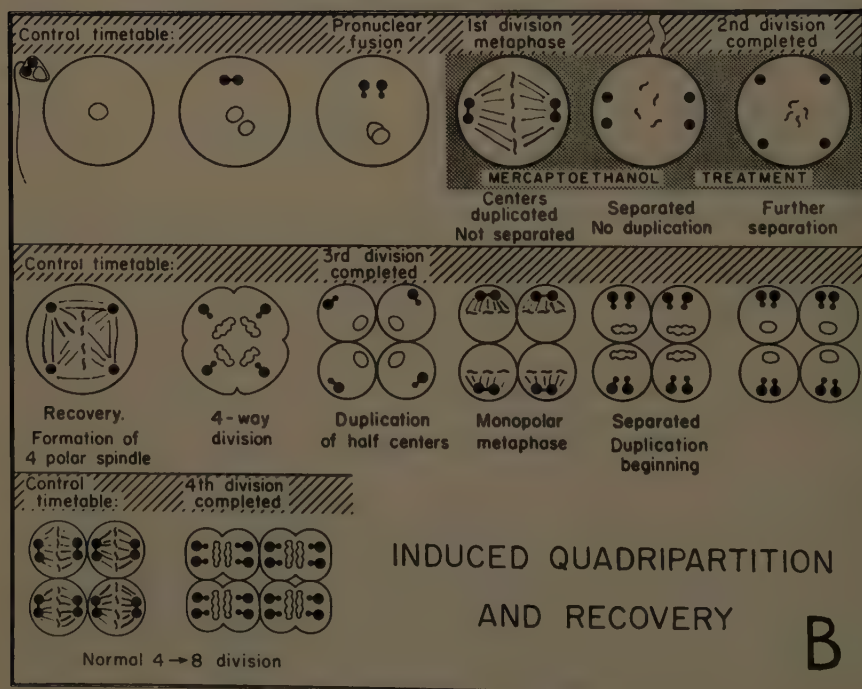
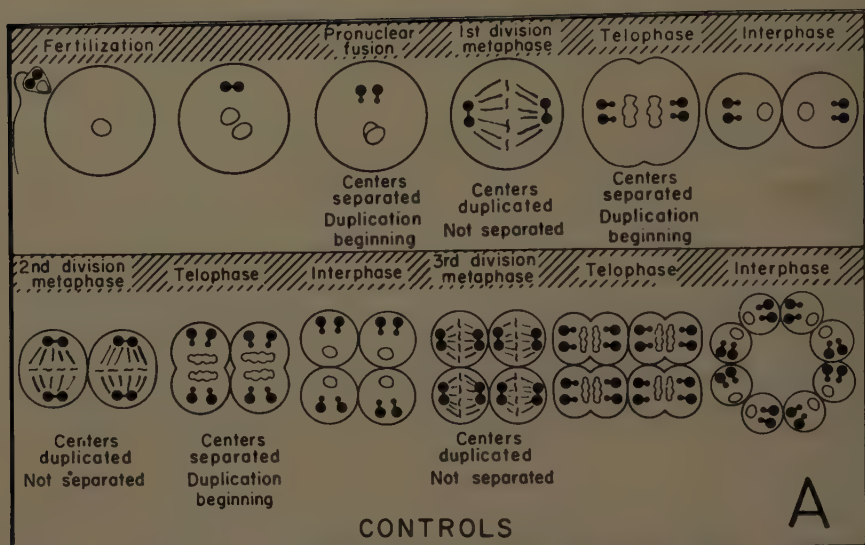


FIGURE 1A. The scheme of normal generative reproduction of the mitotic centers in echinoderm eggs. The "conception" of new centers is indicated diagrammatically as the appearance of small black dots attached to the larger "parent" units. "Parturition" or splitting-apart of parent and daughter units is indicated by the absence of the connecting lines. As the diagram indicates, conception of new units and the splitting part of the earlier generation take place at about the same time in the mitotic cycle.

FIGURE 1B. An interpretive diagram of experiments demonstrating that the centers are duplex structures and that the splitting of centers is distinguishable from the "conception" of new centers by the sensitivity of the former to mercaptoethanol. This experimental design is used to demonstrate the induced four-way splitting of the chromosomes, as discussed in the text. These experiments are described in detail by Mazia *et al.* (1960).

stance, and the kinetochore; these are all thought to play important roles in chromosome function even though they are not currently considered to be part of the primary genome. A fission model of chromosome reproduction would imply that all of these are doubled in conjunction with the duplication of the DNA and histone. A generative model would suppose that the DNA is the self-reproducing portion of the chromosome, that a complete chromosome first produces a second complement of DNA (and probably histone), after which this daughter genome organizes the remainder of a complete and functional daughter chromosome. If such a generative model is applicable, if chromosome reproduction involves a parent-daughter relationship, and if the complete development of the daughter takes time, we might expect to find that chromosomes too were fundamentally duplex. In this formulation, the duplicity would not imply mere genetic redundancy, but also the coexistence of two generations in a chromosome. The parent and daughter strands would ordinarily be inseparable until the daughter had completed its development, and the units that were separated in normal mitosis would each consist of a parent strand and a daughter strand. The idea of a fundamentally duplex chromosome is not a new one; on the contrary, it has been a favored hypothesis of many cytologists and has been based on such phenomena as half-chromatid breaks following irradiation and the actual observation of a resolvable double-strandedness during anaphase (for example, Manton, 1945). By analogy with the experience with centrioles, it should be possible, by delaying division without delaying the development and parturition of the daughter strands of each chromosome, to split a chromosome four ways at a given division. This has actually been accomplished in our laboratory by Thomas Bibring (Bibring, 1960). Sand dollar eggs are blocked at metaphase by mercaptoethanol. As is shown in FIGURE 1B, the centers complete their development and separate to form a tetrapolar figure. When the block is removed, the cell divides into four. During the block, there is no synthesis of DNA by the blocked condensed chromosomes. After the egg divides into four, the daughter nuclei contain only one half the normal amount of DNA at the time of the next prophase, but a count at metaphase shows that the number of chromosomes is the full diploid number. It is possible to explain this only by a four-way split of each chromosome. Since these cells go on to further divisions, it can be concluded that the chromosome sets received a full genetic complement as well as a full number of chromosomes, even though they inherited only one half the normal amount of DNA. It may be added that the full amount of DNA is restored before the next effective division by the fact that the cells have to go through an extra cycle without division in order to restore the required number of centriolar units. This rather complex device is shown in FIGURE 1B. Thus there is at least the possibility that the total reproduction of chromosomes follows a generative model, although this is not proved.

Finally, our aim of analyzing cell division into separate, separable, or dependent processes leads us to ask whether there is an obligatory relationship between the reproduction of the centers. This we have recently analyzed (Bucher and Mazia, 1960). The experimental question was simple. Knowing the time at which the conception of new centers in sea urchin eggs could be blocked by mercaptoethanol (Mazia *et al.*, 1960) we could determine whether

this also blocked the synthesis of DNA. The answer was clearly negative; DNA synthesis proceeded independently of the conception of new centrioles. Therefore the normal sequence of these two reproductive events is not a necessary sequence. These events may proceed in any order, and all that is necessary is that both take place before division begins.

Thus the analysis of the major reproductive events of the cell division cycle has given us information as to the time of synthesis, the numbers of units involved and their possible methods of reproduction, and the dissociability of the two. Much of this analysis was impossible or was at best limited by the uncertainties of microscopic observation a dozen years ago, and has become possible because of new methods and new ways of looking at problems of reproduction. I doubt whether the squid has been moving backwards.

Mobilization of the Mitotic Apparatus

The mitotic apparatus embodies the plan of mitosis. When it is fully organized at metaphase, the destinations of the chromosomes and the avenues by which they will reach their destinations are fully defined. If we look at a metaphase figure, we can say that mitosis consists of the movement of sister chromosomes to sister poles.

The mitotic apparatus is not just a scheme; it is a thing. It can be isolated and, no matter how abnormal the isolation methods may be, they will always assure us that the apparatus has a characteristic chemical unity that differentiates it from the rest of the cell from which it has been isolated.

Let me review briefly some of the newer insights into the mitotic apparatus, confining myself to references to work discussed in this publication.

Chemical nature and quantitative features. Study of the isolated mitotic apparatus assures us that it is composed largely of protein and accounts for a large proportion of all the protein in the cell. The proteins seem to be associated with RNA (Zimmerman, 1960). A few kinds of protein molecules are quantitatively predominant, judging from Zimmerman's (1960) study by physical and chemical methods and Went's immunochemical analysis. The electron microscope (Gross *et al.*, 1958) tells us that the mitotic apparatus, viewed as an oriented gel, excludes the larger particles of the cytoplasm such as mitochondria, but includes, in one form or another, elements of the microsomal fraction.

Origin. We inherit from classical cytology a number of hypotheses and controversies concerning the origin of the mitotic apparatus. One is the problem of nuclear versus cytoplasmic origin at the time of division. Almost certainly this is no problem at all. In a number of instances, the assembly of the mitotic apparatus in the cytoplasm before the breakdown of the nuclear membrane has been seen clearly. An example is the *Haemaphysalis* endosperm studied by A. Bajer and S. Inoué. In this material, it is seen that the spindle is not only forming during prophase but that its components are already orienting with respect to the poles to which the chromosomes will travel. (I regard these observations as evidence of the presence of a centriole-like operation in plant cells). On the other side, the assembly of substance of the mitotic apparatus inside the nucleus before the membrane disappears is strongly indicated

in some kinds of cells. The outstanding example is found in the work of Stich and McIntyre (1958), who demonstrate this by an X-ray absorption method whereby they actually "weigh" and analyze the material assembling in the nucleus of the *Cyclops* egg and show its quantitative and qualitative equivalence to the mitotic apparatus that appears subsequently. Thus we can only refer the question of nuclear versus cytoplasmic origin of the mitotic apparatus to specific cases.

A second question is whether the substance of the mitotic apparatus is synthesized at the time the apparatus appears, or whether it is synthesized as preparation for division and is merely assembled at the time of division. As H. A. Went points out, the latter opinion was strongly held by many of the cytologists of the past, who described an "archoplasm," a fibrous component of the cytoplasm ingathered in the vicinity of the centers at the time of division. This archoplasm was equivalent to what Went describes as a precursor, although it was viewed as a resolvable fibrous system rather than as a pool of molecules. As Went has shown, a modern analysis based on the immunological identification of the proteins of the mitotic apparatus, supports the "preformationist" view. In addition, we may cite the beautiful cytochemical work of Kawamura and Dan (1958), who have visualized the assembly of the mitotic apparatus as a gathering of proteins rich in free-SH groups around the mitotic centers. The work of E. W. Taylor (1959) on the inhibition of the formation of the spindle in newt fibroblasts by chloramphenicol also suggests that the synthesis of spindle proteins is completed some time before the spindle is first seen. Thus we have good reason to believe that the formation of the mitotic apparatus is an assembly of pre-existing units.

The bonding of the mitotic apparatus. If the apparatus is a reasonably coherent assembly of macromolecular units, a major problem of its structure is the problem of intermolecular bonding. In my own work (Mazia, 1959), I have stressed and perhaps overstressed the possible participation of sulfur bonds. At first the emphasis was on S—S bonds, but more recently attention has been turned to other and poorly understood bonds involving protein-SH. The work being reported by Paul R. Gross in this publication provides convincing evidence of a major role of hydrogen bonds, and Norman Anderson has given a perspicacious analysis of the roots of the problem of intermolecular bonding in cell division. I need point out only that such experiments and interpretations, while still very tentative, are an example of the level at which our problems are now being discussed. Perhaps such matters could have been discussed a decade ago, but only a few people who knew about mitosis also knew the language of macromolecular physical chemistry.

Physical aspects of mitosis. A decade ago, the need to study the colloidal aspects of cell division had been clearly expressed and had been embodied in a certain amount of important research, notably by the schools of L. V. Heilbrunn and Douglas Marsland. Problems could be formulated in terms of properties of gels, but these were not well-correlated with specific macromolecules and with the features of mitosis revealed by the morphologists. For example, those who dealt with gels did not deal with spindle fibers, and vice versa. All this has changed, primarily because of the advances in microscopy. Outstand-

ing examples of the newer approach to the physics of the mitotic apparatus are given by S. Inoué, W. Bloom, E. W. Taylor, and R. E. Zirkle in this publication. I need not summarize their experiments, but will essay an over-all impression of their outcome; the mitotic spindle is not merely a gel but a gel upon which it is possible to impose orientation, transforming regions of the gel described by the colloidal schools of thought into the fibers so important to the microscopists' interpretations of mitosis. The orientation is consistently and intelligibly related to the centrioles and kinetochores even though it is highly unstable, and it is reversible.

We must confess that we have not yet come to grips with the central problem of the physical organization of the mitotic apparatus: the specificity of the connections between kinetochores and centers. There is no longer any doubt that the connections exist, and the fact that they are seen as reversibly oriented regions of a mitotic gel makes the problem of specificity all the more difficult. The problem may be formulated in terms of what I may call the exclusion principles stated in a definite way by Boveri in 1904. Each kinetochore is connected to a pole, and sister kinetochores may not be connected to the same pole. There are various interesting hypotheses to describe the establishment of the connections, notably the theory proposed by Östergren (1950), but they do not account for the connections in terms of molecular orientation imposed by the centers and kinetochores over long distances. The answer probably lies in the activity of centers and kinetochores, about which we know absolutely nothing. Part of the problem is to account for the formation of the sub-microscopic spindle filament described by the electron microscopists (Porter, 1955) and its relation to the microscopic spindle fiber that we see so clearly in Inoué's polarization-microscopic records. The easiest and most logical solution is given by the hypothesis of Lettré and Lettré (1959) who postulate that the spindle filament, along with the centrioles and kinetochores, are permanent and self-reproducing units, but definitive evidence for this hypothesis has not yet appeared.

Defining our progress toward an understanding of the mitotic apparatus as an organ of the cell, we can concede considerable advances toward the understanding of the apparatus as a biochemical mass, but must admit little progress toward a comprehension of the specific relations of chromosomes to poles that are the essence of the problem.

Energetics of Cell Division

The unique and most impressive aspect of the energetics of cell division is, of course, the movement of the chromosomes. We may remind ourselves that there is an additional investment of energy currency in the biosynthetic preparations for division, but this tends to be lost in the shuffle of other interphase events. Ever since biologists have been attempting to assess the energetics of life processes by the conventional physiological approach—by measurements of oxidations or of heat production—attention has been given to cell division, and a great deal of work could already be summarized by Brachet in 1947. The over-all outcome of the earlier work was essentially negative but revealingly so; no significant extra expenditure of energy could be associated with cell division. In the more recent period, respirometers of the utmost sensitivity

have exposed small fluctuations in respiration associated with cell division, but even these have been questioned. The controversy has recently been summarized by Zeuthen (1960), to whom we owe so much of the information we have. If we choose not to take sides in the controversy, we can conclude that the period of mitosis in marine eggs, the only material that has been studied thoroughly, is either a period of slightly depressed respiration as compared with interphase or else there is no significant change. This does not necessarily mean that the energy cost of division is negligible. At worst, it would indicate only that the cost of synthetic preparations for division are as high or higher than that of division itself. It could also indicate that the price of division in energy currency is paid before division begins.

The latter point of view has been developed by a number of workers. One line of approach, through the consideration of the role of substrates such as oxygen in the entrance into division of animal cells that are essentially ready to divide, is reviewed by S. Gelfant in this monograph. Perhaps the most stimulating conceptual attack to the problem has been that of M. M. Swann (Swann, 1952, 1957). The essential proposals are: that a controlling event in the division cycle is the filling of an "energy reservoir," that the event precipitating actual division may be the siphoning of that reservoir, and, above all, that the division process itself runs on previously stored energy sources. The experimental evidence for the latter concept is as good as we can expect it to be: no known specific inhibitor of energy-mobilizing systems stops mitosis once it has begun. On the other hand, the results of the search for the energy reservoir in its most literal sense—a potful of high energy compounds—has not always been successful. The one case that could be interpreted as favorable evidence is Plesner's (1958) study of the rise and fall of nucleoside triphosphates in *Tetrahymena* before division. It has also been noted by Swann that the postulated energy reservoir is rather tightly confined into a compartment of the cell specifically associated with a given division; other cell processes cannot draw upon it, and the energy allocated to one division can be drained while the reservoir for the following division is filling.

I propose another way of looking at the energetic problem of division, which is logically simple but not easily accessible to experiment. This proposition is that the mitotic apparatus itself is the energy reservoir! All this means is that the mitotic apparatus is formed as an "activated" system and that, once the energy has been invested in its formation, it can do its work spontaneously. We must remind ourselves that the mitotic apparatus is not like a muscle, which works repeatedly. The analogous system would be a muscle that is formed before each contraction, disperses during or after the contraction, and has to be formed anew every time a contraction is required. We could imagine that such an hypothetical muscle could be formed in its activated state, in which case no further expenditure of obvious energy sources would be involved in its contraction.

Putting aside such speculations, we can at least conclude that the research of the last decade has discovered a basic and unpredicted principle of the energetics of cell division; that the price in energy is paid before the visible onset of division; and that the division process itself is insensitive to a great many of the inhibitors of other cell functions. May we consider this progress?

The Mitotic Movements

The chief mitotic movements have long been accessible to direct observation: (1) the prophase contraction of the chromosomes; (2) the poleward separation of the centers; (3) metakinesis or the movement of the chromosomes into the metaphase configuration; (4) the movement of chromosomes to the poles at anaphase; and (5) the spindle elongation or movement-apart of the poles at anaphase. If we ask immediately about the mechanisms of these movements, we are likely to receive a pessimistic answer. Certainly, we cannot make a factual statement of the mechanism of any of them. However, the field has not remained stagnant. On the contrary, students of mitosis have been taking the proper and scientific first steps toward a clearer picture of the problems: the accumulation of accurate measurements of the movements. A few such measurements existed earlier but, during recent years, we have acquired a vivid and quantitative picture of chromosome contraction in living material (Bajer and Öestergren, 1959), of the separation of the centers (E. W. Taylor, elsewhere in this monograph), of metakinesis (Bajer, 1958), and of the anaphase movements in a great variety of cells (Jacquez and Bieseke, 1954). Since my theme is analysis, I must note that we have useful data not only on the individual movements but also on the coordination of the several movements. What is surprising is that important phenomena that were long accessible to observation, but were overlooked, have been forced on our attention. A good example is E. W. Taylor's observation of the striking shortening of the spindle at metaphase. In my opinion, this observation favors, although it does not prove, the notion that the metaphase status of the mitotic apparatus involves a balance between a "pull" of the still-unsplit chromosomes toward both poles and a "push" in the central spindle, the pole-to-pole element of the mitotic apparatus. This could easily be naïve and incorrect, but it represents the sort of hypothesis that can gain or lose force in the light of the newer detailed quantitative description of mitotic movements.

Still admitting that we know nothing about the mechanisms of chromosome movements, we can cite other piecemeal advances. For example, the accurate plotting of metakinetic movements supports earlier inferences that the chromosomes move into their metaphase positions by means that are related to the anaphase movements; at least, we can say definitely that they move by their kinetochores and that the movement is already under the government of the poles. The classical analogy between mitotic movements and muscle contraction is coming under direct experimental test. The "model" experiments of Hoffmann-Berling (1959) have been most stimulating, although it is still difficult to judge whether the chromosome separation induced by ATP in glycerol-extracted cells is really comparable to normal spindle elongation. Went has described another approach to the question: the immunological comparison of the proteins of muscles and mitotic apparatus of the same organism. In our studies of the isolated mitotic apparatus we have had occasion to compare the proteins with known muscle proteins, with respect both to composition and enzymatic properties.

One more line of progress should be mentioned. Whatever the state of energetic activation of the mitotic apparatus or of the postulated energy

reservoirs may be, the division cycle as a cycle must reflect or be reflected by changes in the dividing cell as a whole. At one moment the apparatus assembles, at another moment it begins to move the chromosomes and, following this, it begins to break down and disappears. The conditions of its stability, instability, and work performance must be governed to some extent by its intracellular environment. What do we know about the mitotic cycle of the cell as a whole, so to speak? The impetus to such studies derives from the classical work of Rapkine (1931), which has been amplified by recent work summarized by Herbert Stern (1959) and by myself (Mazia, 1959). The influence of Rapkine's work has focussed attention on the thiol metabolism of dividing cells. In a sense, Rapkine's view that mitosis is accompanied by a "glutathione cycle" has been confirmed, except that the major SH-carrying molecule in the cycle is probably not glutathione itself. The important study by Sakai and Dan (1958) implicates an SH-polypeptide other than glutathione in sea urchin eggs, and Hase *et al* (1959) have isolated an SH-carrying nucleotide-peptide complex that seems to be involved specifically in cell division in *Chlorella*. In this publication T. W. James describes how the sulfur nutrition of several flagellates may influence the division time without much effect on the total generation time. The problem is to relate the SH-cycle—and other biochemical changes in the cell as a whole accompanying mitotic movement—to the movement itself.

It is not surprising that we can say so little about the mechanism of mitotic movements. What can we say about the mechanism of any form of biological movement? We know more about muscle than about any other system, but at present all we can say about mechanism is that filaments either slide or do not slide. Thus far we students of mitosis are confined to the footsteps of the students of muscle action and of the broader promises of mechanochemistry, studying the structural, molecular, mechanical, and metabolic concomitants of mitotic movements as best we can.

Cytokinesis

Some of us may be more entranced by the purposeful ingenuity of the cell in carrying out the mitotic chromosomal cycle than by the brute fact that, at the end, the cell does usually chop itself into two. Certainly one of the important problems of cell division is the division of the cell. A discouragement to a general attack on cytokinesis has been the seeming diversity of methods of division among various organisms. What is possessed in common by a cleaving spherical egg, a "bubbling" dividing tissue cell, a plant cell that is forming a phragmoplast and cell plate, and a plant endosperm that is chopping itself into cells after a burst of mitosis has been completed? For each of these forms of cytokinesis we have a reasonable store of factual information. For example, about a cleaving echinoderm egg we know such things as its pressure-temperature dependence, the pattern of extension and apparent contraction on the surface, the surface rigidity, the electronmicroscopic structure of the surface, surface birefringence changes, the effects of a number of chemical agents, and the independence from the mitotic apparatus after a

certain stage. We also have an abundance of theories for each kind of cytokinesis, but do not have a unified theory nor are we sure that one is possible.

On the whole, the attack on cytokinesis in animal cells has focussed on properties of the surface, even though the contributions of the internal events were stressed by earlier students (for example, Dalcq and Simon, 1932). However important the surface events may be in the mechanics of division, it is obvious that they must normally be coordinated with the geometry of the mitotic apparatus in some way. The correspondence between the equator of the spindle and the plane of cytokinesis calls for some form of communication between the poles (or the equator) and the surface. Moreover, a great variety of evidence, including the studies by Hiramoto (1956) on the removal of the mitotic apparatus, suggests that the surface receives its message from the spindle at some time during anaphase, after which cytokinesis becomes independent of the mitotic apparatus. Without venturing any positive generalization, I wonder whether several of the contributions to this monograph do not contain the germs of an adequate explanation of these facts and, with such an explanation, a common denominator for plant and animal mitosis.

I refer to the accounts related by K. R. Porter and G. Öestergren on the one hand, and by L. I. Rebhun and A. Zimmerman on the other. Öestergren assigns to the plant spindle an important role in the formation of the new cell wall, by way of a mechanism of alignment of particles at the equator that is related to the mechanism of alignment of the chromosomes. Porter's observations suggest that the material collected at the equator as a phragmoplast and cell plate is part of the endoplasmic reticulum that has come in from the poles. Rebhun describes the collection at the poles of vital-staining vesicles that may conceivably be related to endoplasmic reticulum, and Zimmerman has described how the breakdown of such vesicles may lead to premature cytokinesis. Speculatively, these observations could be unified if we imagine that in both plant and animal cells a part of the cytokinetic mechanism is the formation of new cell surface by the fusion of internal membranous material with the cell membrane. Since cytokinesis is related to the poles, it would be expected that the internal membranes would behave in an orderly way with respect to the poles. In the plant cell, their movement toward the site of the new surface is inward from poles to equator, and we may grant that Öestergren is correct in assuming that their final equatorial alignment is an action of the spindle. In animal cells we are not sure that the membranous component moves at all, but may we not speculate that the accumulation of the vesicles in the asters may be a sign that some of them will ultimately merge with the cell membrane in a polarized way? In the animal cell, perhaps, they join the surface at the poles, behaving like the structure agent once postulated by Swann (1952). All this is rank speculation, and probably wrong, but it seems to me that we have been confronted with a most unexpected relationship between internal membranes and the mitotic apparatus, and it is natural to relate this to the precise coordination of the cell surface and the mitotic apparatus in normal cell division.

Controls and Regulation

This publication presents a number of present day viewpoints on the control of cell division, and I think we can make a number of generalizations:

(1) There is no obligatory, causal relation between the growth of the cell in total mass and its division.

(2) The relation between growth and division may be described in terms of what I may call "the principle of parallel pathways," which has been stated clearly by O. Scherbaum and has been employed by T. W. James in his analysis of cell synchronization. According to this principle, the period between divisions includes a number of specific preparations for division, themselves dissociable from each other. These are distinguishable from growth processes related to the interdivision doubling of biosynthetic mass but not specifically related to division. H. I. Hirshfield and R. Rustad have given us an example in which reduction in cell mass leads only to a partial setback with respect to the preparations for division.

(3) The failure of a cell to divide may be viewed as a block in the preparations for division; the normal tendency of a cell is to divide. We have been shown that the block may be imposed at various points in the pathway to division: H. Quastler describes the common situation where the pathway to differentiation involves the imposition of a block to DNA synthesis, quite soon after the previous division. S. Gelfant deals with quite another situation—epidermal cells have completed their DNA synthesis and are blocked with respect to some other prerequisite to division.

(4) Concerning the nature of the blocking factors, all that we may say with any certainty is that they are external to the cell itself. A. D. Glinos and H. F. Stich describe the conditions of the regulation in various situations, and it would be expected that the cells they are dealing with would all divide if explanted to an environment in which the organismal regulation was missing.

I have not documented these generalizations very thoroughly, but if I did it would be clear that they derive whatever force they have from the methods and the enthusiasms of the contemporary approaches to the cell.

Conclusions

All that I have been trying to say is that we are writing a page in the history of cell biology. It has seemed necessary to insist that it is an interesting and informative page because of the impatience engendered by the intricate beauty and purposefulness of cell division. Who has not been tempted to throw up his hands when watching a film of mitosis? It seems superfluous to say that it is not the last page in the book.

The basic analysis of the reproductive operations of the cell has been successful to the extent that we can dissect these operations into real processes, processes susceptible to clean experimental designs. Our list of these is fairly long and will ramify and grow longer. That is all to the good. Whatever danger exists that we may neglect the integrated wholeness of the network of events by which one cell makes two cells—and it is not so great these days, when biological biology has regained a certain respectability—we cannot ask pointed experimental questions about "cell" division but we can, as I have shown, ask them about the reproduction of chromosomes, the reproduction of the centers, the provision of the substance of the mitotic apparatus, the assembly and orientation of the mitotic apparatus, the energetics of mitosis, the movements of the centers, the movements of the chromosomes, the role of the surface

in cytokinesis, the role of the mitotic apparatus in cytokinesis, the intracellular biochemical environment of the mitotic processes, and the nature of blockage in suppressed division in differentiation systems. The list could be longer. These are the fruits of the analytical approach.

At the same time, we have been able to define and illuminate the basic weaknesses of our present position, notably in conceptions of motor mechanisms in cells, of environmental and organismal actions on the otherwise inexorable progress of cells toward division, and of the specificity of centrioles and kinetochores. This list could be a long one, too.

Given complexity, we may yet avoid turning it into obscurity.

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EXPERIMENTAL INDUCTION OF THE FURROWING REACTION IN THE EGGS OF *ARBACIA* *PUNCTULATA**

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Introduction

It seems well established that protoplasmic sol-gel reactions, which give rise to the spindles, asters, and cortical plasmagel of the cytoplasm, play a very basic role in relation to the complex mechanisms of cell division (Marsland, 1956; Mazia, 1959). However, many unsolved problems remain as to the precise manner in which the formation of these gelated cell components is initiated and regulated. The accurate and orderly sequence of events in cell division would infer that there is an over-all controlling system, but the controlling mechanism is still unknown. Recently studies have been conducted, on sea urchin eggs, that shed new light on this intriguing problem. A new procedure has been found that permits one to study cell division by initiating cytokinesis at almost any time during the mitotic cycle.

It has been established by numerous investigators that before cytokinesis occurs there is a precise series of mitotic events that achieve a distribution of the chromosomes to the daughter cells. However, the new method (Zimmerman and Marsland, 1956; Marsland *et al.*, 1960), which employs high hydrostatic pressure and high centrifugal force, permits cytoplasmic division to proceed without any preliminary nuclear events. The purpose of this study is to investigate the nuclear and cytoplasmic factors that appear to be associated with the induction of the furrowing reaction.

Materials and Methods

Pressure centrifuge. The pressure-centrifuge head was similar to the one described by Brown (1934). A needle valve permits the "sealing in" of hydrostatic pressure on one side of the head, while the control section on the other side contains the specimens at atmospheric pressure. Thus it is possible to subject control and pressurized eggs simultaneously to equal centrifugal forces.

In most experiments the centrifugal force was standardized at 41,000 g. A variable transformer (Variac) placed between the centrifuge and the power supply allowed for the stabilization of any fluctuations in the voltage. In addition, the speed of the centrifuge was checked periodically with a stroboscope.

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† Lalor Research Fellow during a part of this study.

The pressure-centrifuge head was driven by a 0.5 H.P. Dumore motor. Thus, with various belt pulley ratios up to 3.38 to 1 and with a centrifugal radius of 6.8 cm., forces as high as 68,000 g could be obtained. The full build-up time was only 15 sec., and only 8 sec. was required for complete retardation.

A 1 to 2-mm. layer of 0.95 molal sucrose solution was placed at the bottom of both the control and experimental centrifuge chambers. Thus the eggs remained suspended in the sucrose-sea water gradient, and they were prevented from being flattened against the bottom of the centrifuge chamber.

Pressure pump. This pump consists of a modified hydraulic jack capable of generating pressures up to 16,000 lb./inch². A flexible stainless steel pipe conducts the pressure to the experimental chamber, and a pressure gauge, connected to the pump, permits direct reading of the pressure. With each stroke of the pump handle the pressure is built up 2000 lb./inch², so that a pressure of 12,000 lb./inch² could be attained within 6 sec. The pressure can be sealed within the pressure centrifuge by a needle valve and the pressure-centrifuge head disconnected from the pump and then subjected to centrifugal treatment.

Temperature control chamber. Prior to and during the experimental treatment, the eggs were kept in a constant temperature housing that was set at 20° C., showing a maximum variation of $\pm 0.3^\circ$ C. During all centrifugations the refrigerating unit was operating so that the heat generated by the rotation of the motor and the head did not produce any significant elevation of the temperature in the control housing.

Animals. The shedding of eggs by *Arbacia punctulata* was initiated by the intracoelomic injection of 0.5 ml. of 0.53 M KCl or by the electrical shedding method of E. B. Harvey (1952). The animals were inverted over 100 ml. beakers of sea water at 20° C. and allowed to shed for 15 to 20 min. Subsequent to shedding, the eggs were washed twice by decantation with 20° C. sea water. The stock eggs were kept at this temperature pending investigation of successive samples; however, fresh stocks were prepared if an experimental series required more than 4 hours to complete. Sperm was obtained from excised testes and stored in a dry covered stand or dish in the refrigerator. For insemination, two drops of dilute (1 drop of dry sperm to 15 ml. sea water) sperm suspension were added to about 20 ml. of egg suspension.

Staining. In making the Feulgen preparations the eggs were fixed in 10 per cent formalin for 24 hours, washed 3 times in distilled water, and dehydrated through graded alcohols to absolute alcohol. The eggs were attached to microscope slides with 0.5 per cent celloidin-alcohol-ether solution, hardened in 70 per cent ethyl alcohol, rehydrated, and washed 2 times in distilled water. A hydrolysis time of 12 min. in 1 N HCl at 60° C. was employed. The eggs were stained for 1 hour in Schiff's reagent. Subsequent to staining, the cells were washed three times, 10 min. each, in SO₂ water, and then 3 additional times in distilled water. The eggs were dehydrated to 70 per cent alcohol, quickly passed through 80, 95, and 100 per cent alcohol, and then placed in carbol phenol. They were cleared in 2 changes of xylol and mounted in Damar.

In 2 series of experiments the eggs were subjected to lipoid extraction procedures prior to Feulgen staining (cf. Marshak, 1956; Marshak and Marshak, 1955). In these studies, after fixation in the formalin, the eggs were attached

to the microscope slides by embedding them in a thin layer of 5 per cent gelatin hardened over the fumes of full strength formalin for 24 hours at 4° C. After washing and dehydration in graded alcohols the eggs were immersed in a freshly prepared methyl alcohol-chloroform solution (2/1, V/V) for 30 min. After rehydration the eggs were stained by the Feulgen technique as previously described.

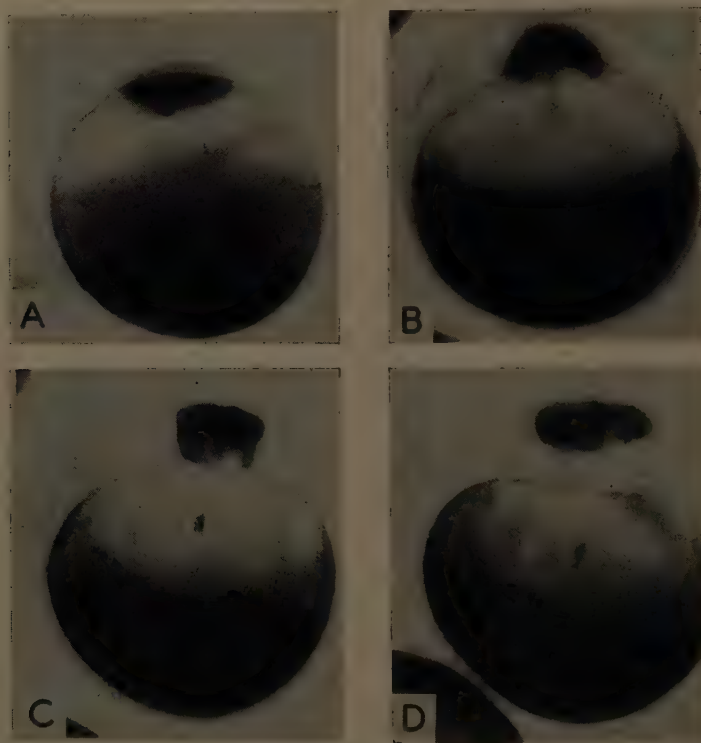


FIGURE 1. Successive stages of an *Arbacia punctulata* egg exhibiting an experimentally induced furrowing reaction. The inducing treatment was a 4-min. pressure centrifugation at 41,000 g at 10,000 lb./inch², initiated 35 min. after insemination at 20° C.: (A) immediately after release of pressure, when the egg is spherical and highly stratified; (B) 3 min. later; (C) 1 min. later; (D) 3 min. later; now the egg shows completed furrow. The "blastomeres" are connected by an elongate strand of hyaline coat material. Reproduced by permission from *Experimental Cell Research* (Marsland *et al.*, 1960).

In a few cases whole eggs were studied by the standard acetocarmine smear technique. Occasionally, whole eggs were stained with Reynaud hematoxylin and cleared in glycerol. In addition to whole-egg studies, some of the eggs were cut into 10- μ sections and stained in standard fashion with Heidenhain's hematoxylin.

Results

Experimental induction of furrowing. At varying times (15 to 55 min.) after insemination, the developing eggs were subjected to the furrow-inducing treatment. This procedure consisted of high-speed centrifugation (40,000 to 50,000

g) at high pressure (8000 to 12,000 lb./inch²) for a short period (2 to 5 min.). Subsequent to such pressure centrifugation, the eggs were kept under continuous observation. Immediately after treatment all the eggs were completely spherical and highly stratified (FIGURE 1A). However, in the following 2 to 5 min., a vigorous furrowing reaction appeared in a large percentage of the cells (FIGURES 1 and 2). The control eggs, in contrast, which had received the same (simultaneous) treatment except for the absence of pressure, did not show any

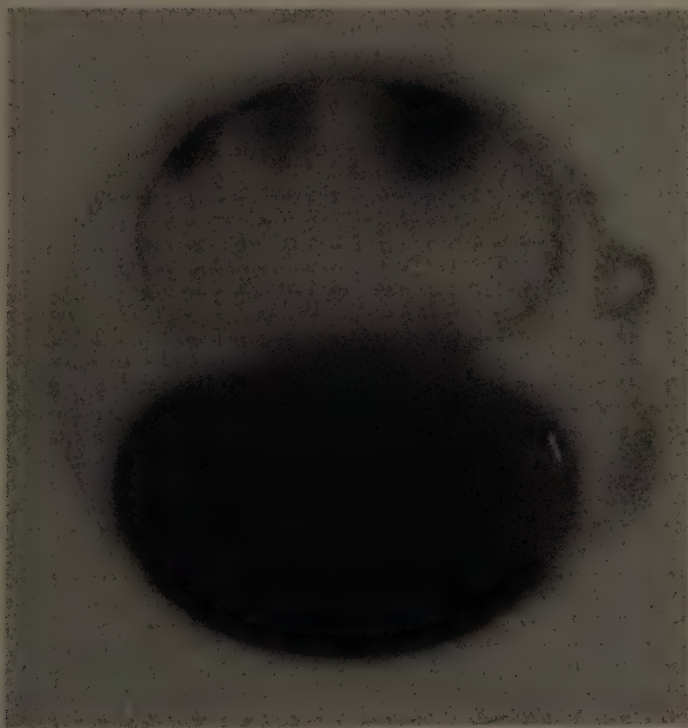


FIGURE 2. Furrow reaction initiated 35 min. after insemination (at 20° C.), that is, 25 min. before normal furrowing appeared in the controls. The inducing treatment starting 26 min. after insemination, was a 4 min.-pressure centrifugation (41,000 g and 12,000 lb./inch²). Immediately after treatment the egg was completely spherical. The photo was taken 5 min. after treatment. The induced furrow divided the egg into 2 "blastomeres" of approximately equal size.

signs of furrowing until the normal time of division arrived. Moreover, the plane of the experimentally induced furrowing was invariably at right angles to the axis of centrifugation, whereas the later furrowing of the control eggs passed through the light and heavy poles of the cell, parallel to the centrifugal axis.

Position of the induced furrows. Although there was no variation in the orientation of the induced furrows, the position or level of the plane of furrowing varied considerably. Sometimes the furrows passed through the equator, midway between the light and heavy poles (FIGURE 2), but more often they were displaced more or less in the direction of the light pole, never toward the



FIGURE 3. Highly displaced induced furrowing in *Arbacia* eggs. Inducing treatment consisted of a 4-min. pressure centrifugation (41,000 g and 12,000 lb./inch²) initiated 35 min. after insemination.

heavy region. Sometimes, indeed, the displacement was very great, so that only the oil cap was pinched away (FIGURES 1 and 3).

Conditions of furrow induction. In general, the susceptibility of eggs to induced furrowing was not related plainly to the stage of mitotic development at the time of treatment. Furrow induction (at 20° C.) could be obtained routinely as early as 15 min. after insemination, and it could be initiated as late as 55 min. after insemination. In one experiment 92 per cent of the eggs, pressure centrifuged 10 min. after insemination, exhibited induced furrowing. This is the time when the monaster has reached full development and the gamete nuclei are fusing. Following syngamy no large fluctuations in induced furrow susceptibility were found until just prior to cytokinesis. Furrow induction late in development is complicated by the occurrence of the furrowing normally associated with the culmination of mitosis. At this time (about 53 min. after



FIGURE 4. Furrowing reaction experimentally induced after first division, but preceding second division. In this experiment the inducing treatment was a 4-min. pressure centrifugation at 41,000 g and 12,000 lb./inch². Note that the induced furrow is initiated at right angles to the centrifugal axis.

insemination) the nuclear membrane undergoes spontaneous dissolution, and a high percentage of experimental inductions can be obtained by centrifugation alone, that is, in the absence of high pressure.

Experimentally induced furrows were also obtained in the two-celled stage, prior to the onset of the second division. Such induced furrows were essentially similar to those seen in the one-cell stage. The plane was always at right angles to the centrifugal axis. In some batches of eggs, furrows appeared near the equators (FIGURE 4), dividing each egg into four fairly equal segments, but in others they were displaced centripetally, resulting in the formation of two small and two large blastomeres.

The process of systematically varying the centrifugal force and duration and the hydrostatic pressure was undertaken in order to ascertain the relations of these factors to the induction phenomenon.

The studies that employed a systematic variation of centrifugal force, while maintaining a standard pressure and centrifugal duration, were unrewarding.

Centrifugal forces less than 40,000 g very seldom induced furrowing, although both hydrostatic pressure and centrifugal duration were maintained at maximal levels. Unfortunately, it was not possible to centrifuge at forces higher than 41,000 g, since this force was near the tensile strength of the pressure-centrifuge head.

By studying the frequency of induced furrows at different pressure levels it was possible to evaluate the role of high hydrostatic pressure in relation to furrow induction. It was soon established that high pressure was an essential part of the inducing treatment, except in the special situation when the treatment was initiated very late in the mitotic cycle. The greatest frequency of furrow induction occurred consistently at the higher pressure levels (12,000 lb./inch²). However, most batches of eggs studied showed greater than 90 per cent furrow induction at 8000 lb./inch² when the centrifugal time was extended to 4 to 5 min. When pressures below 7000 lb./inch² were employed, induced furrows were scarcely ever observed even with extended centrifugation times.

In addition to high pressure and centrifugal force, the duration of centrifugation is also an important factor in the induction phenomenon. A series of measurements were conducted 35 min. after insemination in which the duration of centrifugation was systematically varied while both the force (41,000 g) and the pressure (12,000 lb./inch²) were kept constant. As illustrated in the accompanying graph (FIGURE 5) the frequency of induced furrows increases in a sigmoid fashion as the centrifugal duration increases. In centrifugation times below 60 sec., less than 10 per cent of the cells exhibited furrows and, at 30 sec., there was virtually no furrow induction. On the other hand, furrow induction under these same pressure and force conditions approached a maximum after 4 to 5 min. centrifugation, when almost all the eggs (90 to 100 per cent) exhibited the induction reaction.

Induction in relation to the spindle-aster complex. No trace of spindle or asters could be demonstrated in the eggs during or immediately prior to the appearance of experimentally induced furrows, provided the inducing treatment involved a pressure of 8000 lb./inch² or more. This was true for fixed and sectioned material and for whole eggs fixed and stained by the acetocarmine technique, even when the inducing treatment was initiated late in the cleavage period, at which time spindles and asters could readily be demonstrated in the control eggs. Undoubtedly the pressure treatment caused a complete solution of the spindle-aster complex (when present) and, without question, induced furrowing can occur in the complete absence of these gelated structures. Later, however, when the experimentally treated eggs spontaneously resumed their subsequent cleavages, spindles and asters were readily demonstrable.

In the *special case* where eggs received an inducing treatment late in the mitotic cycle (after the fifty-third minute at 20° C.) high pressure was not essential for induction, that is, centrifugation alone sufficed to induce furrowing in a high percentage of the eggs. It was possible to differentiate such induced furrows from naturally occurring furrows, since the induced furrows appeared slightly earlier. Moreover, the induced furrowing plane was always at right angles to the centrifugal axis. When an inducing treatment without pressure was initiated late in the first cleavage period after the mitotic apparatus had

been formed, it was clearly evident that the orientation of the displaced mitotic apparatus was entirely random. There was no regular relationship between the plane of the induced furrowing and the orientation of the mitotic apparatus. This is in accord with the important observations of E. B. Harvey (1935).

The nuclear factor. In highly centrifuged *Arbacia* eggs, of course, there is a

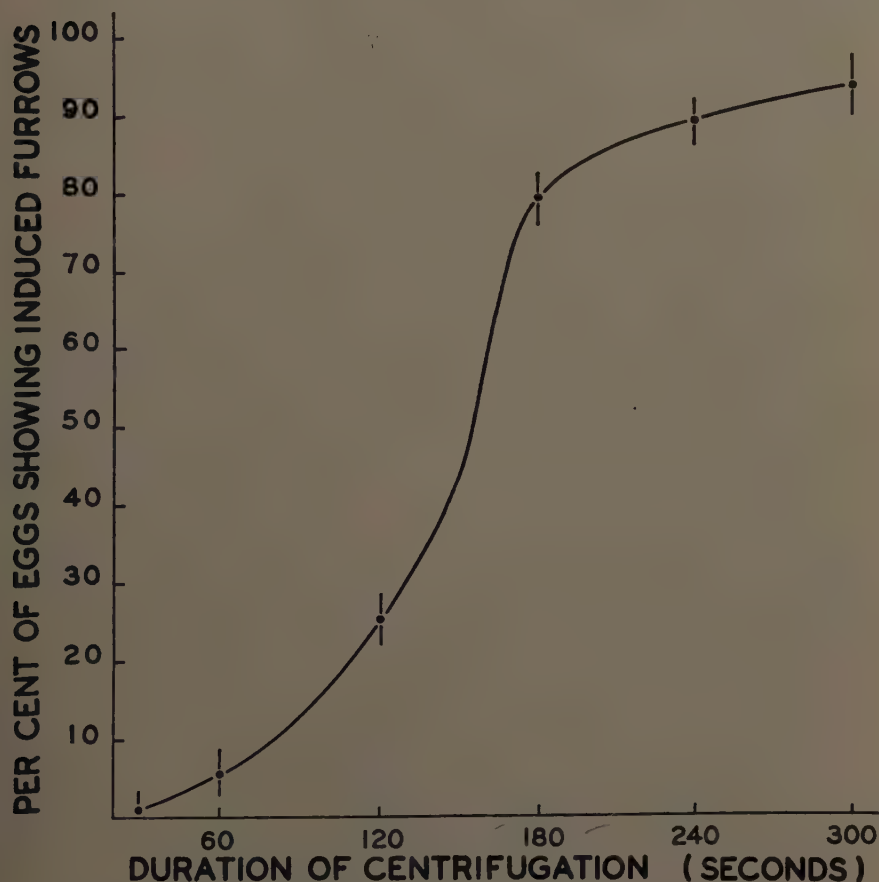


FIGURE 5. The effect of duration of pressure centrifugation on the frequency of induced furrowing in *Arbacia*. Furrowing was initiated 35 min. after insemination (at 20° C.) by pressure centrifugation (12,000 lb./inch² and 41,000 g). Reproduced by permission from *Experimental Cell Research* (Marsland *et al.*, 1960).

very distinct stratification of the cellular components. Below the centripetal oil cap the nucleus lies in the hyaline zone, followed by the mitochondria, yolk, and pigment zones (FIGURE 6B). Such a pattern was always seen in the control, nonpressurized eggs, provided that mitosis had not progressed to the stage where the nuclear membrane had undergone dissolution, in which case, of course, whole nuclei could not be seen. On the other hand, nuclei were never seen in any pressure-centrifuged eggs, provided that the treatment was sufficient to induce furrows (FIGURE 6A).

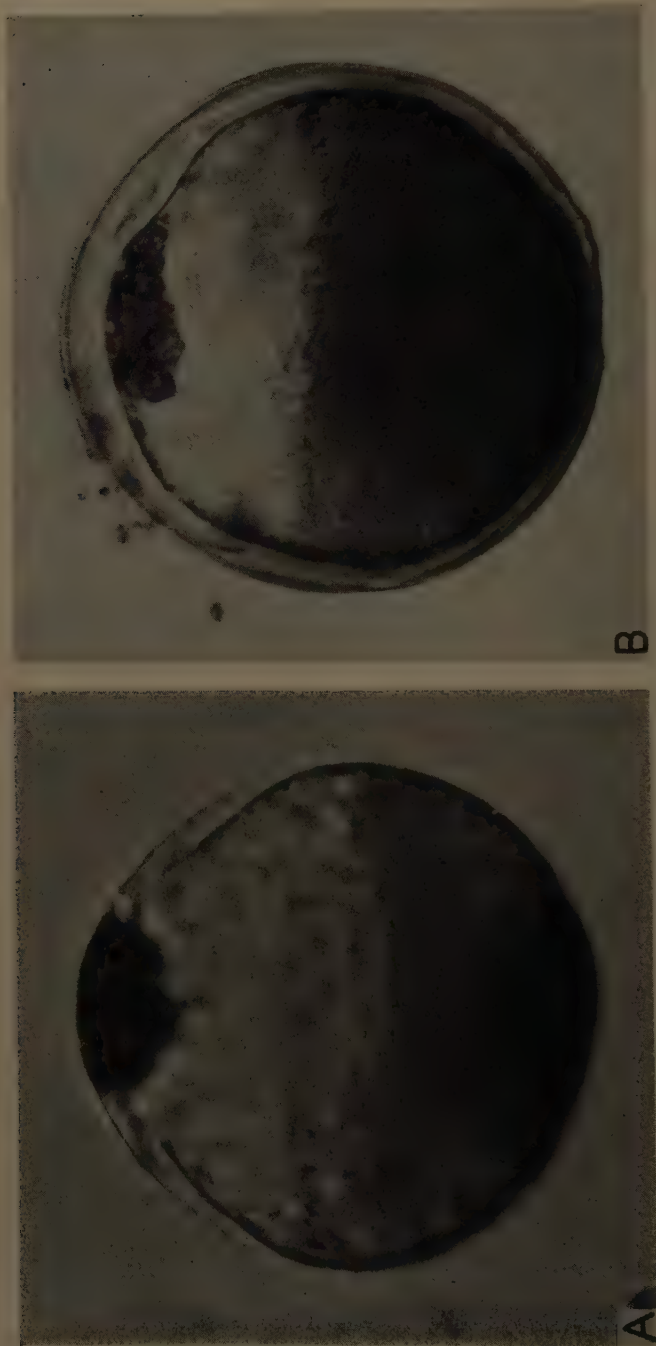


FIGURE 6. Photograph showing 2 eggs (*Arbacia punctulata*) centrifuged simultaneously for 4 min. at 41,000 g at 20° C. The experimental egg (A) centrifuged at 12,000 lb./inch² does not have an intact nucleus. It shows displacement of all pigment vacuoles from the cell cortex and a distinct pigment zone at the centrifugal pole. The control egg (B) centrifuged at atmospheric pressure displays an intact nucleus in the hyaline zone and the presence of pigment vacuoles in the cortex; it does not display a distinct pigment zone. Five minutes after treatment virtually all the pressurized eggs displayed induced furrowing. Reproduced by permission from *Experimental Cell Research* (Marsland *et al.*, 1960).

From our studies on living and fixed material it is evident that the inducing treatment disrupts the nuclear membrane, causing a breakage of the large zygote nucleus. In addition, the data leads one to suspect that such nuclear rupture plays an essential role in the furrow-induction phenomenon.

Immediately after pressure-centrifuge treatment, the eggs were examined critically. Samples of treated eggs were placed in fixative and, subsequently, sectioned and stained, while another sample was permitted to develop while observed under a phase microscope. In neither the living eggs nor in the eggs stained with Schiff's reagent, Reynaud's hematoxylin, or acetocarmine was it ever possible to demonstrate intact nuclei subsequent to an adequate pressure centrifugation. Instead, one could always find small clumps of Feulgen-positive material (FIGURE 7). The position of these clumps varied from cell to cell. In eggs fixed and stained after the induced furrow had already cleaved the cell

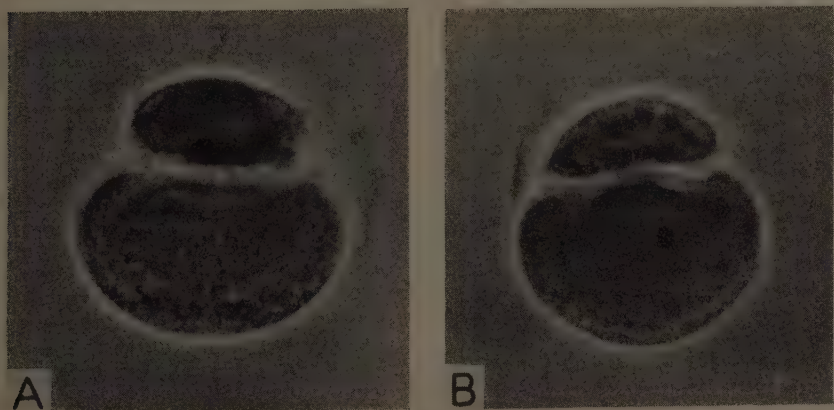


FIGURE 7. Eggs fixed after completion of induced furrowing and stained with Schiff's reagent. Note the absence of the intact nuclei. The Feulgen-positive material may lie in either the light (A) or heavy (B) "blastomere." The eggs were fixed 7 min. after centrifugation. Reproduced by permission from *Experimental Cell Research* (Marsland *et al.*, 1960).

into heavy and light blastomeres, the Feulgen-positive material could be found in either the light or the heavy blastomere. Furthermore, there was no consistent relationship between the position of the Feulgen material and the level of the plane of furrowing (FIGURE 7).

In eggs subjected to more moderate pressure-centrifuge treatment not adequate for an induction of the furrowing reaction, an intact nucleus generally could be seen clearly in the well-defined hyaline zone. Frequently such nuclei were slightly elongated and, as established in eggs stained with acetocarmine and Reynaud's hematoxylin, there was a distinct intranuclear stratification. The stained material (presumed to be chromatin material) appears to be relatively heavy, since it occupied a centrifugal position in the nucleus. Nonpressurized centrifuged eggs, on the other hand, never showed much, if any, intranuclear stratification. Apparently high pressure exerts a solating action on the karyoplasm (see also Pease 1941 and 1946) and, certainly, pressure increases susceptibility to nuclear fragmentation.

The cytoplasmic factor. In addition to nuclear rupture, at least one other factor localized in the cytoplasm appears to be involved in the process of furrow induction. Recently it has been demonstrated that certain cytoplasmic components are associated with the division process (Marsland, 1958). These cytoplasmic components are the β granules that have been identified in various eggs (Dalcq *et al.*, 1956; Rebhun, 1958). The β granules lie within small cytoplasmic vacuoles (Marsland, 1958) and they exhibit metachromatic staining properties in the presence of toluidine blue and other vital dyes. Also, it is possible to follow the movements of these granules during the mitotic cycle (Mulnard *et al.*, 1959).

Involvement of both nuclear and cytoplasmic factors. The following experiments indicate that, in addition to nuclear rupture, a breakage of the cytoplasmic vacuoles with subsequent disintegration of the β -granule material is also essential for furrow induction.

The relatively large nuclei of eggs in the first cleavage period were found to be susceptible to breakage by rather moderate pressure centrifugation (for example, 8000 lb./inch² for 90 sec.), a treatment not adequate to rupture the β -granule vacuoles or liberate the β -granule material. Such eggs with ruptured nuclei but unruptured cytoplasmic vacuoles did not ever display any cleavage induction. However, the smaller nuclei of the two-cell stage were found to be more resistant to rupture than the β -granule vacuoles and, in this case, it was possible to choose a pressure-centrifuge treatment (10,000 lb./inch² for 120 sec.) which would break the vacuoles but not the nuclei—and again no induction occurred. In other words, experimentally induced furrowing has been found to occur only when a rupture of both the nucleus and β -granule vacuoles has been achieved.

Stability of the induced furrows. In general, about 70 per cent of the induced furrows were successful in the sense that they cleaved the egg completely and did not recede subsequently. Incomplete furrows, in contrast, generally receded prior to or during the formation of the subsequent naturally induced furrows.

Eggs receiving pressureless centrifugations just prior to the normal division time showed vigorous furrows at right angles to the centrifugal axis. Then frequently a second set of furrows, oriented quite haphazardly with reference to the first set, began to appear within 3 to 4 min., usually before the first set had finished cleaving the egg. Preliminary investigation indicates that this second set of furrows is determined by the orientation of the centrifugally displaced mitotic apparatus, which tends to be quite random. Generally, the second (naturally induced) furrows, which frequently went through to complete cleavage, tended to supersede the first (artificially induced) furrows. However, many irregular cleavage patterns were observed that resulted in 2-, 3-, and 4-celled embryos, often with blastomeres of grossly unequal size.

Further development. In general about one half of the experimental and control eggs were kept for observation of their further development. A small percentage of apparently normal free swimming embryos, including some well-developed plutei, were obtained from most of the experimentally (pressure-centrifugation) treated specimens. However, almost every batch contained many abnormal embryos. Some of the abnormal embryos were "half embryos,"

that is, only one of the first blastomeres continued to cleave. Some did not develop beyond the 4-, 8-, or 16-cell stage, while others proceeded to the blastulae stage and some became exogastrulae. Some embryos appeared to be half-sized but quite normal, except for an amorphous remnant of an uncleaved blastomere still adhering to one end.

The magnitude and duration of the pressure treatment also had a distinct effect on subsequent development. Very few, if any, normal embryos developed when pressures above 10,000 lb./inch² were applied for more than 3 min.

Discussion

The dissolution of the nuclear membrane during mitotic prophase, which regularly foreshadows the appearance of a cleavage furrow in most animal cells, forms a natural basis for the idea that one or more substances released from the nucleus may play a role in triggering off the cleavage reaction. However, experimental evidence in support of this hypothesis has been scant or lacking hitherto, and quite a few observations have seemed to contradict this view. The present experiments, on the other hand, give basis for proposing that both nuclear and cytoplasmic factors participate in the process of furrow induction.

Perhaps the strongest evidence against the view that nuclear material is involved in cleavage is provided by the work of E. B. Harvey (1936 and 1938). This study shows that parthogenetic merogones of *Arbacia* are capable of cleaving despite the absence of nuclei. It should be noted, however, that furrowing in this case is delayed, feeble, and irregular. It does not occur until about 4 to 5 hours subsequent to activation. By this time one or more cytasters have appeared in the "cell," and it seems likely that the cytasters may be involved in initiating the furrowing reaction. Perhaps β -granule material, synthesized in an unusually large amount during the long lag, is carried to the cell surface by the asters. Here a feeble furrowing reaction might be initiated, although very little nuclear material were available. It must be remembered, however, that some nuclear derivatives must be present in the merogonic cytoplasm, liberated into the cytoplasm by the breakdown of the large germinal vesicle during maturation. In the many experiments reported here, no induction of furrowing was ever obtained, unless the nuclear membrane lost its integrity, either as a result of pressure centrifugation, or by virtue of its normal dissolution during mitotic prophase.

Nuclear and cytoplasmic participation. The migration and distribution of vitally stained granular material around the nuclei and developing mitotic mechanism has lead to the hypothesis that this material plays an important role in cytokinesis. Investigation of metachromatically stained β -granules by Dalcq and his collaborators (1956), Rebhun (1958), and Mulnard *et al.* (1959), in the cytoplasm of different eggs have shown that these granules are gathered around the asters and carried to the cell surface just prior to the onset of furrowing.

An association of metachromatic granules with division recently has been demonstrated by Kojima (1959), who has shown, in three different species of sea urchin egg, that cytoplasm granules, vitally stained with toluidine blue, Nile blue sulfate, Janus green, or neutral red, distribute themselves around the developing asters and are related to the "cleavage activity" of the cell. Kojima

also observed that when fertilized and unfertilized eggs are broken by centrifugation, only the half containing the vitally stained granules retain their ability to cleave.

In the present investigation we have observed that the metachromatic granules reside within vacuoles and that only when the experimental treatment is sufficient to break the vacuoles and release the granules, which subsequently disintegrate, do we obtain conditions favorable to furrow induction. In addition we have found that the integrity of the nuclear membrane must be destroyed either experimentally, as a result of centrifugation or, naturally, by spontaneous dissolution.

Timing of furrow induction. In the *Arbacia* it has been demonstrated that the furrow reaction, which normally does not occur until telophase (58 min. after insemination at 20° C.—cf. Zimmerman *et al.*, 1957), can be induced prematurely, starting as early as 12 min. after insemination. Accordingly, it seems likely that the substances that are concerned with the initiation of cleavage are not synthesized entirely during the first cleavage period but are available in adequate amounts soon after maturation, or even earlier.

Under normal conditions the cleavage furrow appears about 8 min. after the dissolution of the nuclear membrane. The similar timing in normal and experimental eggs suggests the possibility of transport of active "cleavage material" to the cell surface. Perhaps centrifugation augments and accelerates the transport of this material, since induced furrows occur quickly, that is, 3 to 5 min. after treatment. In the present investigation the observations of Dalcq *et al.* (1956) and others have been confirmed. Undoubtedly β granules are carried to the polar regions as the asters grow, and it seems probable that nuclear (cleavage) substances could also be carried to the cell periphery in similar manner. A likely candidate, therefore, for distribution of the nuclear as well as cytoplasmic components to the cell cortex might be the mitotic mechanism.

As a result of their extensive birefringence studies, Swann (1951) and Swann and Mitchison (1958) have proposed that an "X substance," released from the chromosomes, diffuses to the surface where it initiates furrowing. On the other hand, it seems possible that the β -granule material might be able to reach the cell surface even in the absence of a mitotic apparatus—by virtue of the unusual, radially directed movements described by Rebhun (1959).

The present experiments indicate that furrow induction depends upon at least two factors, one released from the nucleus and the other from the β -granule vacuoles of the cytoplasm. Moreover, it is proposed that the mitotic apparatus, although playing a vital role in normal mitosis, perhaps in directing the movements of cleavage factors, is not completely essential for furrowing. In fact, several workers have reported that cleavage can occur in the absence of the mitotic apparatus. The original observations by Chambers (1938) and more recently by Mitchison (1953) seem to demonstrate that cleavage can occur after the asters are destroyed by mechanical agitation with microneedles. Likewise, the treatment of eggs with colchicine (Beams and Evans, 1940; Swann and Mitchison, 1953) or with irradiation (Tahmisian, see discussion by Inoué, 1952) prevents aster formation but allows the cells to cleave. Also, Hiramota (1956) has observed that cleavage may take place in sea urchins from which the spindle and asters have been removed by sucking them into micropipettes

during anaphase. However, furrowing rarely occurs when the spindle aster complex is removed from the cell during metaphase.

Site of furrow induction. It is generally agreed that the peripheral cytoplasm of the egg, which is also called the cell cortex and the peripheral plasmagel layer, represents the focal region of furrowing activity, although the precise mechanism of furrowing remains problematical. Accordingly it seems likely that the focal region for furrow induction is likewise in the cell cortex, particularly at the poles, and there is some evidence to support this view.

Although experimentally induced furrows sometimes cut through the equator of the cell, more frequently they are displaced more or less into the light hemisphere, never into the heavy yolky part of the centrifuged egg. Sometimes, indeed, the displacement is so drastic that only the oil cap is cleaved away. Thus it seems reasonable to believe that if there are substances responsible for furrow induction, they would be distributed along the axis of the centrifugal gradient. All the available evidence suggests that the density of both the nuclear and cytoplasmic factors is relatively light compared to the hyaline cytoplasm; hence they would tend to be displaced toward the light pole, in the region of the oil cap.

The density of the nucleus as a whole appears to be about that of the clear hyaline cytoplasm since, in centrifuged *Arbacia* eggs, the nucleus comes to rest in the hyaline zone. Pressure-centrifuged eggs also give evidence of intranuclear stratification, the heavier Feulgen-positive material lying centrifugally and a clear material occupying a centripetal position. When the nuclear membrane is ruptured by high-pressure centrifugation the lighter unidentified nuclear material would be expected to pass centripetally and the heavier stained material centrifugally.

The metachromatically stained β granules in the cytoplasmic vacuoles are also relatively light. In centrifuged eggs they come to rest primarily in the hyaline zone, although a few may also be found in the mitochondrial zone. When the fertilized eggs are pressure-centrifuged the vacuoles rupture and the metachromatic material, which under normal conditions is microscopically resolvable in the form of red granules, is no longer visible. Instead, one finds a diffuse, nongranular reddish coloration in the cytoplasm, and this diffusely stained material is most concentrated at the centripetal portion of the egg in the region of the oil cap.

The foregoing observations suggest the hypothesis that the low density materials of the cytoplasm and nucleus have their site of action at the centripetal pole, where they initiate the furrowing reaction when the eggs are subjected to high-pressure, high-speed centrifugation. In the case of normal division, on the other hand, it seems evident that the nuclear and cytoplasmic materials are transported by the spindle-aster complex to both poles, so that furrowing occurs at the equator, equidistant between the poles. This viewpoint is supported further by the observations of Swann and Mitchison (1958) and of Inoué and Dan (1951) that very distinct optical changes occur in the polar regions of the egg cortex during telophase just a minute or two prior to the appearance of the definitive furrow. However, the proposal of Swann and Mitchison that an "X substance" released from the chromosomes is responsible for the initiation of the furrowing reaction is not supported by the present evi-

dence, especially the observation that the level of induced furrowing is not determined by the position of the displaced Feulgen-positive material derived from the ruptured nuclei.

Summary

The furrowing reaction in *Arbacia*, which ordinarily does not occur until the telophase of mitosis, can be initiated much sooner, before any sign of nuclear division has appeared. The method of furrow induction consists of subjecting the fertilized eggs to centrifugation at high force (40,000 to 50,000 g) and high pressure (8,000 to 12,000 lb./inch²) for 2 to 5 min. Following such treatment the eggs are quite spherical, but 2 to 5 min. later they display furrows that usually succeed in cleaving the egg completely and irreversibly. Prior to prophase the pressure factor is essential for induction; subsequently centrifugation alone may suffice. The plane of such experimentally induced furrows is always at right angles to the axis of centrifugation. Sometimes the furrows come in at the equatorial level, but more often they are displaced more or less into the light hemisphere of the egg.

Successful induction appears to depend upon two factors, both consequences of the high-force, high-pressure centrifugation: First, the treatment must rupture the nucleus and, second, it must break a number of cytoplasmic vacuoles, each containing several granules that stain metachromatically (red) with toluidine blue. These are the so-called β granules, previously observed by many workers in a wide variety of eggs. Presumably the pressure acts by solating the gel structures of the cell, permitting easier rupture and allowing for a more complete separation and stratification of the cellular components. The results are interpreted in terms of the hypothesis that induction of the furrowing reaction normally depends upon the transport of materials, of both nuclear and cytoplasmic origin, to the polar regions of the cell cortex. Normally both poles are involved and, perhaps, the mitotic apparatus represents the transporting agency. The experimentally induced reaction, on the other hand, seems to involve only the centripetal pole, and the transport is achieved by centrifugal force.

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CELL DIVISION V. MOLECULAR MECHANISMS

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The sequence of movements that comprise the mitotic ballet appears superficially to be the product of many different mechanisms, some of which are dissociable from the rest. When cell division is viewed at the level of molecular interactions, however, it is evident that the same general principles may underlie the formation of structures that are microscopically quite different (Anderson, 1956*b*, *c*). The central problem is that of structure that can produce movements. While in theory particles may be made to move by diffusion forces without intervention of contractile systems (Landahl, 1942), the major movements associated with cell division appear to be structure dependent. In this paper we shall consider first some of the possible mechanisms involved in forming structures that might be capable of visible movement, and then relate these mechanisms, in so far as is now possible, to intracellular events. The difficulties involved in showing that a mechanism that is demonstrable *in vitro* actually operates in the living cell are enormous. However, unless intracellular mechanisms can be studied in isolated systems relatively little detailed information will be obtained.

The mechanisms involved in structure formation from which we have to choose are listed in TABLE 1. The most direct and simple is that involving the synthesis of specific proteins or other macromonomers exactly where and when needed. While problems of structure formation may thereby be solved, the difficulties of controlling synthesis and of positioning synthetic machinery then arise. The mitotic apparatus at least does not appear to be synthesized completely from low molecular weight substances when it becomes visible since the available data suggest no marked rise in protein synthesis during prophase (Prescott, 1957). Rather, the mitotic apparatus appears to be assembled from pre-existing macromolecules (Mazia, 1959; Went, elsewhere in this monograph).

To bind together previously separate molecules requires some alteration conducive to association. Most often invoked in the past as the primary change is the unrolling of globular proteins to give peptide chains. It must be said that there is now little evidence supporting involvement of this mechanism in muscular contraction. Nevertheless the rolling and unrolling of polypeptide chains has been invoked to explain a variety of cellular activities (Monne, 1948). No demonstration of large molecular shape changes by a cellular protein under physiological conditions has thus far been observed. While this mechanism for altering proteins as a prelude to reversible structure formation cannot be completely ruled out, it will not be considered further here.

The removal of a group shielding active sites on protein molecules is a mechanism well known in the activation of enzymes and in the fibrinogen-fibrin conversion (Ferry, 1954). That it may be involved in intracellular processes

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is suggested by observation of an increase in acid-soluble nitrogen after addition of calcium to egg homogenates (Gross, 1954). Alternate mechanisms for changing the surface of macromolecules include: complexing with either inorganic or organic ions (usually di- or multivalent), the addition or removal of positively charged groups by transamination, and removal of negative charges by decarboxylation.

A broad spectrum of valencies may link the altered molecules together. Weaker linkages (electrostatic, hydrogen, and other secondary forces) require no special mechanisms in order to be formed. Primary valencies with the possible exception of disulfide linkages generally require the intervention of enzymic catalysis.

TABLE 1
MECHANISMS INVOLVED IN STRUCTURE FORMATION AND MOVEMENT

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|--|---------------|
| (1) Production of macromonomers | |
| (a) Protein synthesis | |
| (b) Nucleic acid synthesis | |
| (2) Alteration of macromonomers prior to interlinking | |
| (a) Unrolling of globulins to yield peptide chains | |
| (b) Removal of groups shielding stereospecific sites | |
| (i) Splitting off of peptide fragments | |
| (c) Addition of groups producing interlinkage sites | |
| (i) Addition of inorganic or organic ions | |
| (ii) Transamination reactions resulting in increased or decreased positive charges | |
| (iii) Decarboxylation or deamination | |
| (3) Interlinking of macromonomers | |
| (a) Mechanisms | |
| (i) Primary valencies | } { Enzymatic |
| (ii) Secondary valencies | |
| (b) Types of polymers | |
| (i) Single species (linked chains or beaded chains) | |
| (ii) Mixed species | |
| (iii) Macromer-micromer polymers | |
| (iv) Chains of multimolecular particles or beads | |
| (4) Movement | |
| (a) Molecular shape change | |
| (b) Chain configuration change with no molecular shape change | |
| (c) Movement of chains by each other | |
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The structures formed may involve polymerization of one, or more than one, macromolecular species. Alternatively macro- and micromonomers may form the linear structures required. It should be noted that the principles involved are not strictly limited to the level of single molecules. Multimolecular rolled chains could conceivably unroll to give beaded chains, while polymolecular particles may aggregate to form ordered structures.

Given fibrous structures, movement may result from shape changes by ad-linearated molecules, by chain configurational changes that do not involve appreciable molecular shape changes, or by movement of chains one along the other. The forces invoked have generally been considered to arise from changes in the number (or distribution) of charges along chains giving rise to alterations in the amount of repulsive force along the chain. When these repulsive forces diminish, the chain would be expected to assume a more coiled,

kinked, and also more probable configuration (Katchalsky, 1954). An alternate mechanism involves the "melting out" of ordered areas to give a more random and contracted configuration (Flory, 1956; Tobolsky and Brown, 1955; Mandelkern *et al.*, 1958). While certain parts of cell division have been thought to involve mechanisms analogous to those operative in muscle, the present state of knowledge concerning muscular contraction provides us with no obvious models that can be applied to cell division. In this state of ignorance it is reasonable to inquire whether any of the mechanisms discussed here and listed in TABLE 1 are actually involved in any phase of cell division.

It has been proposed that chromosomal condensation is due to configurational changes in DNA molecules (Anderson, 1956*b*). The evidence cited centers chiefly around the close similarity between the shapes of the curves relating DNA viscosity to both pH and salt concentration, and parallel changes in the volume of isolated rat liver nuclei subjected to the same variables. The conclusion was drawn that a decrease in viscosity was indicative of tighter coiling of the DNA molecules, a view supported by the parallelism between the viscosity changes observed in synthetic acid polymers and the contraction or elongation of fibers made from the same polymers (Kuhn *et al.*, 1950).

The interpretation that the decrease in DNA viscosity below pH 5 is due to collapse of a semirigid coil appears to be correct (Alexander and Stacy, 1955; Reichmann *et al.*, 1953). The cause is probably both a decrease in electrostatic repulsion along the chain and breakage of hydrogen bonds (Shooter, 1957).

One objection to this simple concept as applied to salt effects arises, however. DNA double helices are rather "stiff" molecules in solution. When viscosity measurements are extrapolated to zero concentration and to zero shear rate, it appears that salts affect chiefly the degree of interaction between separate molecules rather than the shape of individual molecules (Conway and Butler, 1954; Shooter, 1957). The conclusion is based on rather long extrapolations. Both alterations in the size of the random DNA coil and changes in the degree of interaction between individual DNA molecules probably occur. The difficulty lies in attempting to decide what proportion of the effects observed is due to each. While the explanation for changes in the physical properties of DNA solutions have undergone some revision, the original conclusion (Anderson, 1956*b*) that the physical properties of isolated nuclei reflect the properties of DNA appears valid.

Changes in nuclear volume (and in the physical properties of DNA) cannot be directly related to chromosomal condensation until more is known about the organization of DNA in the chromosome. In the interphase nucleus, nucleoprotein appears to be bound in a three dimensional gelwork that does not go into solution when nuclei are torn in dilute salt solutions and that also exhibits a yield value (Anderson and Fisher, 1960). Conceivably DNA molecules could be attached by one end to a protein backbone, as suggested in an early model of Taylor (1957), in such a manner as to give rise to nuclear volume changes without markedly affecting chromosome coiling; or DNA could be linearly arranged as part of an ultrafibrillar structure. It is important, therefore, to know whether DNA exists merely as a series of arboreal appendages, or whether it is responsible in part for the continuity of chromosomal ultra-

fibrils (Schwartz, 1958). If the latter is true it appears more probable that changes in DNA molecules are responsible for chromosomal condensation.

The nature of forces holding atoms or molecules together may be inferred from the conditions required to dissociate them. If, in the nucleus, DNA molecules are bound end-to-end by protein or other substances, and if these strands are cross-linked to give a gel structure, it might be supposed, if linear integrity is important, that the end-to-end linkages would be stronger than the side-to-side interchain linkages. If this is true, then conditions should be sought that might break only the weaker side-to-side linkages. Experimentally, it was found that fresh rat thymus nuclei in 1 *M* NaCl gave rise to a solution of extraordinary viscosity, far exceeding that of a solution (Anderson, 1953; Fisher *et al.*, 1959a) containing the same quantity of purified DNA. As shown in FIGURE 1, when an attempt is made to prepare an extract containing the concentration of DNA often used for viscosimetric measurements (~0.1 percent) an unmanageable preparation is obtained.

Is this viscosity due to the presence of very long strands of DNA interlinked with protein, or is it due to the presence of an expanded three dimensional gelwork? If the latter were true the viscosity should drop markedly at a concentration where the "gel islands" are no longer in contact. Furthermore, a typical "multihit" viscosity versus X-ray dose should be observed. Experimentally, no sudden decrease in viscosity is found with dilution even at concentrations as low as 0.0009 per cent DNA, and with X-irradiation a "one-hit" curve is observed with dilute preparations (Fisher *et al.*, 1959b). (The slight shoulder observed at higher concentrations may be due to a trace of residual gel structure or to radiation-protective substances present in the tissue.) The results therefore support the view that the high viscosity observed in fresh 1 *M* NaCl extracts of nuclei is due to the presence of extremely long strands that may dissociate further at higher salt concentrations. The extreme sensitivity to X rays adds additional support for this view. As little as 10 roentgens produces an easily measurable effect. Studies on the depolymerization of purified DNA, on the other hand, generally require doses in the kilo roentgen range (Shooter, 1957).

Recent studies on the effect of heat on the 1 *M* NaCl extracts show them to be more sensitive than purified DNA (Fisher and Burger, 1960). The energy of the bonds broken appeared to be approximately 20 kilocalories, more suggestive of protein denaturation than rupture of nucleotide chains (FIGURE 2). The rapid and complete loss of viscosity after addition of small amounts of deoxyribonuclease provides additional evidence against the concept of a long protein backbone with DNA molecules attached at intervals.

Studies on structure persisting in extracts are open to the criticism that the structure may have been formed during the extraction procedure. Conceivably DNA and protein (or other molecules), previously separate in the nucleus, could associate on extraction in a new and highly specific manner to give very long fibrils. Means were therefore sought for producing small changes in nuclei that could be assumed to have little effect on individual DNA or protein molecules but which might break, inside the nucleus, very long linear aggregates, if such were actually present. The experiments were based on the common observation that multistranded threads, as for example sewing thread,



FIGURE 1. Nucleoprotein from rat thymus in 1 *M* NaCl. Concentration of DNA ~ 0.1 per cent. Prepared by mixing suspension of nuclei in 2 *M* NaCl.

although held together by very weak forces, do not generally break by breaking these weak bonds. Rather, the main fibers break because their strength is less than the sum total of the weak frictional forces between fibers. If very long multistranded bundles of DNA-protein fibrils exist in the nucleus, it might be possible to produce breaks by merely deforming the nuclei. This was done by placing thymus nuclear suspensions in a Waring Blendor and blending for various lengths of time (Anderson and Fisher, 1960). It was noted that nuclei either remained spherical or were very elongated and remained so. Lack of intermediate shapes suggests that the nuclei exhibit a yield value which, if exceeded, results in strand breaks and permanent nuclear deformation. The

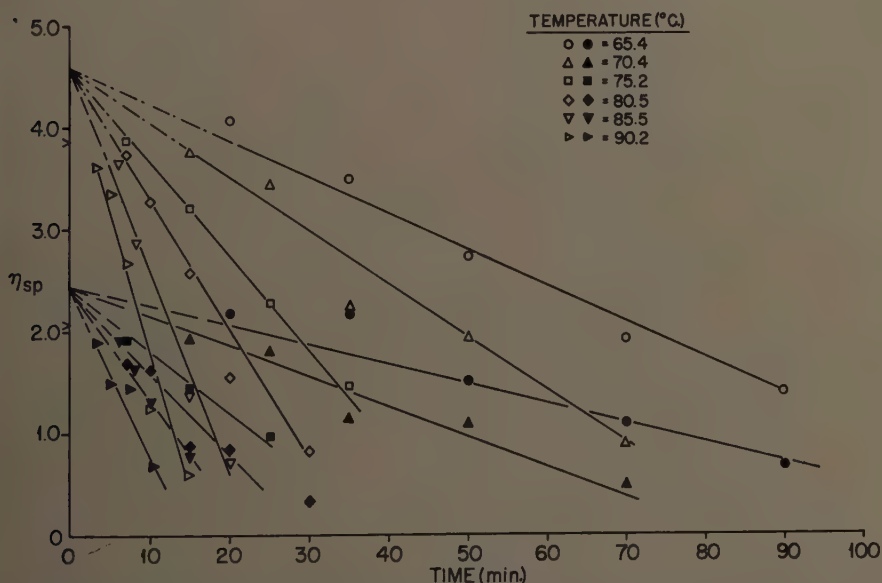


FIGURE 2. Effect of heating at various temperatures on the apparent specific viscosity of a nucleoprotein extract. Upper set of curves for a concentration of 33.5 $\mu\text{g.}$ of DNA/ml.; lower set for 23.0 $\mu\text{g.}$ of DNA/ml. $\bullet$ indicates viscosity of unheated sample (Fisher and Burger, 1960).

viscosity observed on 1 *M* NaCl extraction with sheared nuclei (FIGURE 3) was directly proportional to the number of round, undamaged nuclei remaining. The results could not be explained by the release of nucleases or proteases during blending since the viscosities did not decrease after extraction as would be expected with enzymic degradation. The available evidence therefore suggests that chromosomes contain long strands composed of DNA molecules linked end-to-end by another substance, most probably a protein. Changes in either the degree of coiling of individual DNA molecules or in the degree of interaction between different DNA segments of the long strands may be expected therefore to have profound effects on chromosome volume and coiling.

If the problem of condensing chromosomes at prophase is chiefly one of condensing long strands composed of DNA interlinked and partially coated

with protein, the question then is: By what physiological mechanisms can such strands be condensed? The simplest way is to discharge negative groups on DNA with a polyvalent cation. It was suggested previously that an increased amount of histone or a smaller basic polypeptide, or a simple polyamine (such as spermine, spermidine, cadaverine, or putrescine) might be responsible (Anderson, 1956b). Support for this view was soon forthcoming from a rather

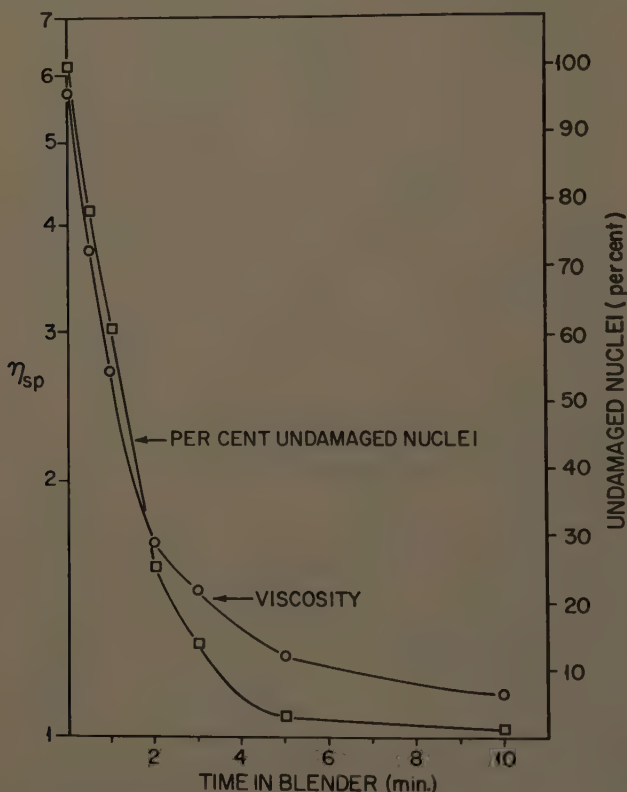


FIGURE 3. Effect of shearing thymus homogenates in 0.14 *M* NaCl in a Waring Blender on the viscosity of subsequently prepared 1 *M* NaCl extracts and on the number of intact nuclei remaining. Note that viscosity observed is proportional to the number of undamaged nuclei.

surprising source. Viral DNA in a number of instances is complexed with polyamines (Ames *et al.*, 1958). These appear to be responsible for condensing the DNA prior to the deposition of the exterior protein coat. Interference with polyamine metabolism after phage infection has been shown not to interfere with synthesis of virus protein and DNA but to prevent formation of complete viruses (Kay, 1959). Agmatine, also a strong divalent organic cation, has been shown to accelerate prophase in grasshopper neuroblasts (St. Amand *et al.*, 1960). The condensing effect of polyamines on isolated nuclei strongly resembles prophase condensation (Anderson and Norris, 1960). Of greater in-

terest is the recent observation of Davidson (Davidson and Anderson, 1961) that polyamines cause overcondensation during cell division in onion root tips and that after division the chromosomes in the daughter cells remain condensed for at least three hours. The fact that the cells completed division suggests that the compounds were not very toxic in the concentrations used and that the effects were due to an exaggeration of the normal condensing mechanism. It is of considerable interest, in view of the suspected relationships between sulfhydryl compounds and mitosis, that methionine is important in polyamine synthesis, and that spermidine appears to be linked to glutathione in *E. coli* extracts (Dubin, 1959).

Thus it now appears possible to unravel the mechanism of chromosomal condensation. This is based on the discovery of methods for obtaining in solution threads thought to be the ultimate strands of the chromosome and on predicted effects of basic substances on the DNA sections of these strands.

We now turn to less well-explored ground: the mechanisms involved in the formation of the mitotic apparatus. These do not appear to involve large shape changes at the molecular level. The chief decision that must be faced at the outset before beginning experimental work in this field is the choice of a model system to investigate. Unfortunately elucidation of molecular mechanisms requires that sufficient amounts of the reactants be isolated for complete biophysical characterization and for studies on reconstituted systems.

The theory of a unitary response by the cell to a variety of conditions has been defended by Heilbrunn (1956) and others. This view holds that sol-gel changes occurring during amoeboid movement, in response to a wide variety of physical, mechanical, and chemical stimuli, during the surface precipitation reaction, the first stages of cytolysis (Loeb, 1913) and *during cell division* are all fundamentally the same. It is not possible to evaluate this theory until the mechanism in one instance is known, nor to prove it until the mechanisms in two or more of these experimental conditions have been elucidated. We therefore chose a system that is to us the most amenable to experimentation. If the unitary hypothesis is correct we are examining mechanisms similar if not identical to those operative in the cell. If it is incorrect we are exploring cytolysis in the experiments to be described.

The soluble proteins of rat liver (Anderson, 1956*a*, the soluble phase) are unstable to incubation *in vitro* at body temperature (Anderson, 1957); a much higher rate of precipitation is observed when the temperature is raised further (FIGURE 4). When the precipitation at 40° C. was followed at short intervals by turbidimetry, a lag was observed (FIGURE 5) suggesting that some preliminary step was necessary before precipitation began. Since ATP disappears rapidly in isolated systems it was considered that degradation of this compound may be a first step. Addition of ATP to the isolated system prevented precipitation for a period of time, after which the rate was faster than in the control. The length of time that precipitation was prevented was proportional to the amount of ATP added. The soluble phase of rat liver is not able to maintain its ATP level by glycolysis (Le Page and Schneider, 1948), and addition of cofactors important for glycolytic activity decreased the rate of precipitate formation in our liver system by about one half. These observations suggest

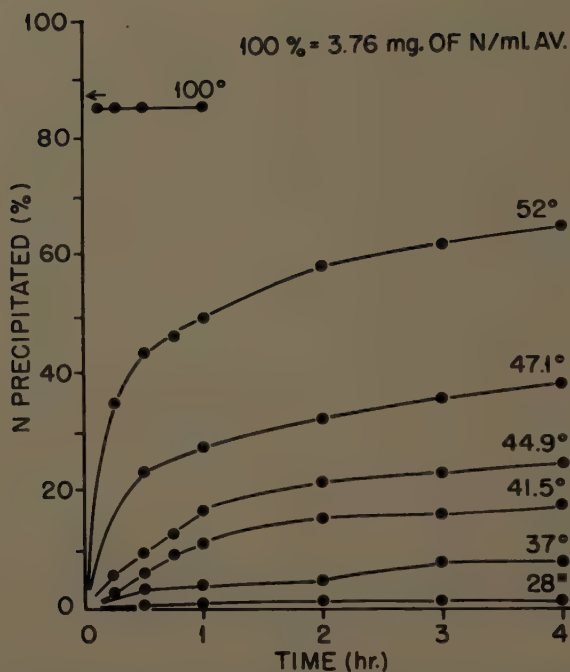


FIGURE 4. The effect of incubation of rat liver soluble protein solution for various periods of time on the per cent of total nitrogen precipitated. Amount precipitated at 100° C. is very close to amount of nondialyzeable nitrogen indicated by arrow.

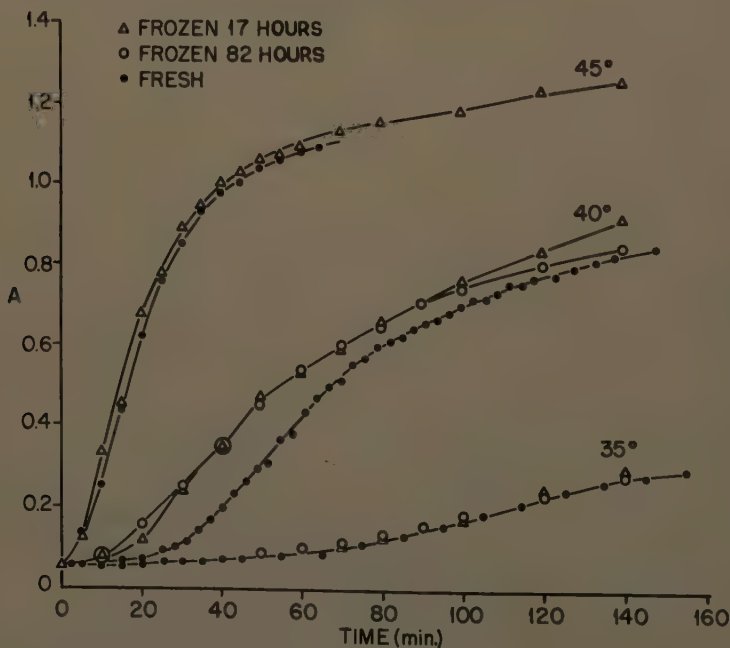


FIGURE 5. Effect of incubation of frozen and nonfrozen rat liver soluble protein solution on the absorbancy measured at 625μ. Note longer lag in appearance of turbidity in unfrozen preparation at 40° C.

that the level of ATP in the cell may be important in preventing *in vivo* the precipitation here observed *in vitro*.

Le Page (1949) noted that the degradation of RNA in liver breis was greatly retarded by ATP. This important observation suggested that RNA may also be important in the system here described. Experimentally, addition of RNA markedly reduced the rate of precipitate formation, while degradation of the "soluble" RNA present by ribonuclease has the opposite effect. The RNA effect suggested that other polyanions might also be effective. This led to the discovery that heparin and polymanuronic acid sulfate ("Paritol") had a protective effect (FIGURE 6) in excess of that of any other compound studied

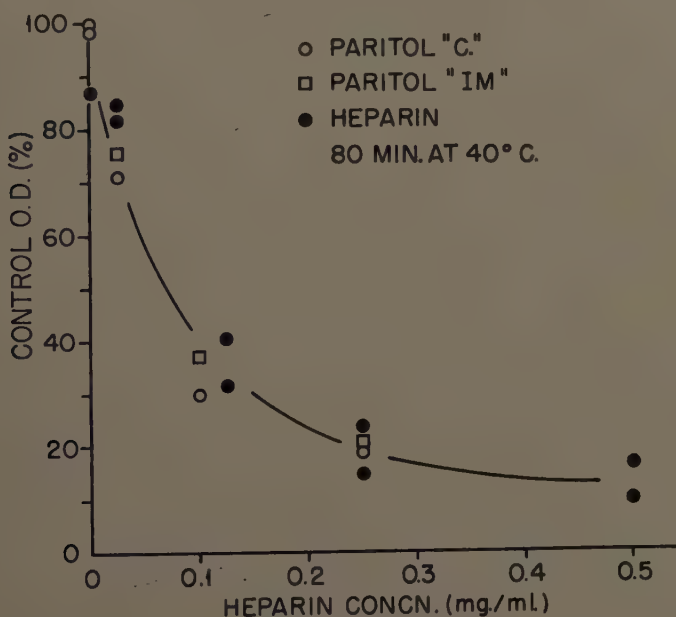


FIGURE 6. Comparison of the effect of heparin and Paritol on the formation of turbidity of rat liver soluble protein solutions incubated at 40° C. for 80 min. Results expressed as per cent of absorbancy of control preparation.

(Anderson *et al.*, 1961). It appears therefore that polyanions generally prevent precipitation of liver soluble phase proteins *in vitro*.

Heat precipitation of albumin is prevented by nucleic acids (Greenstein and Hoyer, 1950). However, the results appear to be due to a solubilizing effect of the nucleic acid rather than by prevention of damage to the protein. To determine whether heparin in our experiments was merely preventing aggregation of altered proteins and not preventing those events that alter the protein molecules, heparin was added at various times after incubation was begun. Only a very slight indication of reversibility was observed. It therefore appears that polyanions may prevent the initial protein alterations rather than inhibit aggregation.

The relationship of these findings to intracellular conditions is fascinating

indeed. Heparin has long been known to inhibit premitotic gelation (Heilbrunn, 1956) and to prevent cytoplasmic sol $\rightarrow$ gel changes. The effect in the cell may be partially due also to RNA, which may be displaced from ribosomes by heparin (Anderson and Wilbur, 1950). Of special interest is the conclusion of Runnstrom (1949) that glycolysis in marine eggs is insufficient to maintain the normal state of the cytoplasm. A decrease in intracellular ATP appeared to give rise to a coarsening of the cytoplasmic ground structure. These findings and relationships suggest that the simple precipitation observed *in vitro* may be an extension of normal *in vivo* processes, and this appeared to justify further exploration.

It should be noted in passing that if cytoplasmic proteins were positively charged, as has been suggested (Heilbrunn, 1952), the polyanions here studied would be expected to have a precipitating effect by themselves. Actually the charge on soluble phase proteins may be demonstrated most easily and directly by free electrophoresis. At pH 7.5 the median mobility of the soluble phase proteins of liver, kidney, testis, and brain are -2.0 , -3.0 , -4.3 , and -5.2 T units, respectively. Only in liver and kidney does one observe a fraction of the proteins with a slight positive charge. Why such large interorgan differences should exist is not known. However the observation that the average mobility of soluble cytoplasmic proteins is directly related to nuclear volume (Anderson and Swanson, 1961) has suggested a new approach to differentiation (Swanson and Anderson, 1961).

Since calcium has been causally related to sol-gel changes (Heilbrunn, 1952), its effects and those of magnesium and spermine were examined. Calcium markedly increases the rate of precipitation and also abolishes the initial lag period. Magnesium has a similar effect. Spermine in very small amounts produces an immediate precipitation in the cold. When this precipitate is removed by centrifugation at low temperature and the clear supernatant incubated at 40°C ., a marked increase in the rate of precipitate formation was observed. These findings are an extension of the pioneering work of Hultin (1950*a*, *b*) and of Gross (1952, 1953, 1954, 1956) who showed that calcium had marked flocculating properties when added to egg or liver breis.

However, in contrast to effects observed with whole breis, no drop in pH with calcium addition was observed. Since a large fraction of the liver soluble phase proteins are precipitated by simply lowering the pH to 4.9 (Anderson, 1956*a*) it is important to note that the precipitation during incubation at 40°C . studied here is not accompanied by a drop in pH.

In confirmation of the observations of Gross (1954) an increase in nonprotein nitrogen, and acid soluble tyrosine and ninhydrin positive material was observed during incubation, suggesting that enzymatic degradation of certain proteins had occurred. It is not unlikely that specific peptide groups are split off certain cytoplasmic proteins, exposing sites capable of binding together previously soluble proteins. If the split-off peptides remain undegraded and can be reattached to the parent molecule, then the reaction would probably be reversible. However if the peptides are degraded to amino acids the polymerization may not be easily reversible, and in a short time formation of additional bonds may bind the proteins even more tightly. It is noteworthy that this appears to be the case with the formation of clots by plasma. The

clot as initially formed can be dissolved by urea, however upon standing, other (including disulfide) bonds make the clot insoluble except under extreme conditions (Ferry, 1954). This stepwise process is a reasonable model for the so-called "unitary response" of the cytoplasm that exhibits reversible early steps, but can, if overextended, lead to precipitation and death.

What is now required is the isolation of the enzymes, substrate proteins and early split products. This work, now in its earliest stages, is being carried out as a part of the Oak Ridge Cell Fractionation Project.

Since it has been proposed that the mitotic apparatus is composed chiefly of one species of protein (Mazia, 1959), it was important to discover whether a single protein was involved in the precipitate formation described in liver. Ultracentrifugal comparison of the same preparation, with and without incubation at 40° C. for 2 hours, showed that material was missing from both of the main peaks previously described in the rat liver soluble phase (Anderson and Canning, 1959). Electrophoretic analysis (Anderson and Anderson, unpublished) showed that at least seven peaks lost material during incubation. No single protein therefore appears to be involved.

It has been suggested that as much as 30 per cent of the proteins of the rat liver cell are involved in the formation of the mitotic apparatus (Swann, 1959). There is no single protein that accounts for this percentage of the liver cell. It is not unlikely therefore that a number of proteins are involved in the formation of spindle and aster. Unfortunately the proteins of the isolated mitotic apparatus have not been examined in sufficient detail to know whether a single protein predominates. The published ultracentrifuge patterns were taken too early in the run and strongly resemble published patterns of the entire soluble phase of rat kidney taken shortly after reaching operating speed (see FIGURE 1; also Anderson and Canning, 1959). Adequate electrophoretic analysis will require prolonged electrophoresis runs over a wide pH range. Recent work on the serology of the mitotic apparatus (Went, 1959) shows that at least two cytoplasmic constituents can serve as precursors of the mitotic apparatus.

The only cytoplasmic structure-forming mechanism that we have been able to demonstrate appears to involve the removal of groups shielding stereospecific sites, and the interlinking of proteins through secondary valencies. The splitting off of shielding groups appears to be catalyzed by polycations.

The relationship between the experiments on cytoplasmic proteins reported here, and changes occurring during cell division remains to be established. Since cell division must eventually be described in terms of well characterized proteins and reactions that can be studied *in vitro*, it is important that the tedious process of sorting out the constituents of the cytoplasm be given serious attention. Evidence suggesting that many of these may fall into discrete groups or families is published elsewhere (Anderson and Canning, 1959; Anderson and Swanson, 1961).

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HEAVY WATER INHIBITION OF CELL DIVISION: AN APPROACH TO MECHANISM*

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Since the discovery of deuterium by H. C. Urey in 1932, a large literature has been produced by investigators who have observed its effects upon biological systems. Urey recognized that isotopic substitution might have especially profound effects upon biological systems,¹ and G. N. Lewis made one of the earliest studies² on deuteration of various organisms. While interest in this problem never declined seriously, the difficulty of obtaining reasonable quantities of heavy water (D_2O) was an eventual restriction upon the scope of biological experiments. Reports appeared steadily, however, if slowly, so that by the early 1950s, 200 or more papers dealing with biological and biochemical effects of deuterium had been published.

Recently, in connection with its use in reactor technology, mass-produced heavy water has become available at reasonable cost. This has led to a significant renewal of interest in the biologic effects of deuteration. This newer literature reflects not merely the older, exploratory interest in isotopic substitution but also the rapid growth of the theory of isotope effects in rate processes, as well as a more sophisticated view of the role played by hydrogen bonds in the three-dimensional organization of proteins and nucleic acids.

Important recent contributions from the investigators at the Argonne National Laboratory, Lamont, Ill.,^{3,4,9} and from the group at the Berkeley Radiation Laboratory, Berkeley, Calif.,^{5-7,9} have re-emphasized observations, reported in some of the earliest papers, concerning the special sensitivity of the cell division process to deuteration of the organisms. Of course, extensive deuteration of cells and organisms affects other things than mitosis, as would be predicted from chemical principles. The large chemical literature on deuterium isotope effects has been ably reviewed by Wiberg,⁸ and newer results are to be described in a future number of these *Annals*.⁹ However, the early findings of Lucké and Harvey¹⁰ on sea urchin eggs, and of Ussing¹¹ on amphibian eggs, have received confirmation from a number of recent studies on both simple and complex systems; a careful reading of these reports makes it clear that even in as complex a system as the mammal, cell division is the process that is often most profoundly inhibited.

This would by itself be interesting, although not necessarily encouraging, since we suffer from an embarrassment of riches in the field of antimetabolites. The difficulty is that with a few notable exceptions (uncouplers of oxidative phosphorylation,¹² the thiol reagents studied by Mazia and his co-workers,^{13,14} and nucleic acid antimetabolites) we do not know the mechanism of inhibition.

Our ignorance of the mechanisms of action of so many chemical antimetabolites

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justifies the careful study of mitotic blockade by heavy water. D₂O is, after all, a simple reagent as compared with, for example, colchicine or even urethane. One cannot necessarily predict that its effects will be simple; indeed, the mass difference between D and H leads to the prediction that primary and secondary isotope effects will be large, and hence that the rates of all reactions in which bonds to hydrogen are affected will be changed to some extent. However, the inhibition of mitosis by D₂O in marine eggs has features that simplify the problem considerably. First, as to absolute magnitude: the "isotope effect" on mitosis is very large; indeed, it can be infinite if defined as a ratio of "rate constants" for cleavage, k_D/k_H , since in 99+ per cent D₂O, the cells do not divide at all. Such an enormous effect could hardly be predicted from the known effects of isotopic substitution in ordinary reactions. Normally, an explanation is offered as follows: whereas the rates of individual metabolic reactions will be changed (usually decreased) only somewhat, the cell's over-all metabolic steady state will be so upset as to produce profound depression of growth and of other function. The inhibition of mitosis in marine eggs is, however, a favorable case for analysis. It will be shown that D₂O exerts its effects with such speed, and at such stages of cell division, that interference with metabolism could hardly provide a suitable or unique mechanism.

Other possibilities do, however, suggest themselves from the data, and these concern interference with normal hydrogen bonding in certain critical systems. Whether this explanation is correct or incorrect, its appearance in the data, unbidden, as it were, leads us to believe that the study of mitotic inhibition by heavy water can have value for the investigation of cell division beyond a mere addition to the already swollen list of mitotic poisons. A brief report of our observations has appeared in another journal,¹⁵ and it may be consulted for certain results not included in the present discussion.

Methods

Eggs and sperm of the Woods Hole sea urchin *Arbacia punctulata* were obtained in the small quantities needed for these experiments by electrical stimulation of mature, gamete-bearing males and females, essentially according to the method of Harvey.¹⁶ Eggs and sperm from the annelid *Chaetopterus pergamentaceus* were obtained by cutting parapodia from individuals with ripe gonads and permitting the gametes to be extruded into sea water. Eggs from both species were washed several times with filtered sea water.

All experiments in which deuterated media were employed were run with two controls so that, in every case, eggs or embryos were followed simultaneously in three solutions:

(1) Sea water control: ordinary sea water, from the running supply in the laboratory, filtered and used at once.

(2) Reconstituted control: the salts from a measured volume of normal sea water, dried *in vacuo*, and redissolved in the same volume of distilled water, the solution being prepared and stored together with the third solution.

(3) D₂O-sea water: the salts from a measured volume of sea water dried *in vacuo* and redissolved in the desired mixture of D₂O (99.5+ per cent) and H₂O, the final volume being the same as that from which the salts were originally obtained.

The heavy water was obtained as 99.5+ per cent material.* Since mixtures of D_2O and H_2O attain an equilibrium among the three species HOH, HOD, and DOD, the "concentrations" henceforth reported refer to the volumes per cent of D_2O used in making the solutions.

Eggs were fertilized and allowed to develop in a variety of vessels but, in any experiment, all samples were kept in identical glassware, with the same degree of aeration in each. The temperature was kept constant throughout each experiment for all samples.

Observations on the several cell populations in an experiment were made as follows: at an appropriate time, a drop of cell suspension was removed from each vessel and placed on a microscope slide marked off into a number of rectangles. As many as ten drops could be accommodated on such a slide, and the sampling could be completed in a few seconds. The slide was then placed quickly on the stage of the microscope and each drop examined and photographed one or more times on 35 mm. film. For counting, it proved convenient to photograph several fields showing between 25 and 50 cells. Ordinarily, this procedure required no more than 3 or 4 min. for the entire slide, frequently a much shorter period (that is, when there were only 3 or 4 samples). The egg populations were sampled and photographed at intervals of a few minutes throughout the period of observation. Where embryonic development, rather than the early cleavages, was the object of study, the samples were observed and photographed at longer intervals.

The photomicrographs were made with a Zeiss LgOG microscope with trinocular change-over device, equipped with an Exakta camera body. The emulsions used were Kodak Panatomic-X and Adox KB-14 developed to high contrast. The records were kept as 36-exposure, 35-mm. filmstrips, and these were later projected with the aid of a filmstrip projector onto a white surface, in a darkened room, for counting. This procedure minimized the statistical difficulties often encountered in making counts of fertilization membranes, spindles, and cleavage furrows, and it provided, in addition, objective records of the cells, to which reference could later be made for such detailed cytological information as is available from observation of the living cell.

The stratification rate for cytoplasmic inclusions in normal and deuterium-treated cells was measured by the centrifuge technique devised and applied by Heilbrunn.¹⁷ W. L. Wilson assisted us in these observations.

Electron micrographs were made from ultrathin sections of the eggs fixed and handled as described by Gross *et al.*,¹⁸ and Delbert E. Philpott assisted in the electron microscopy.

Results

Test of reconstituted sea water. The reconstituted (H_2O) sea water tends to be alkaline, immediately after preparation, to normal sea water. In all of our experiments, these solutions were prepared within a few minutes of the corresponding solutions containing D_2O . The vessels were then stored for a period (usually a few days) sufficiently long to permit the pH in the H_2O -reconstituted sea water to return to within 0.2 pH units of normal sea water. The pH change is a result of re-equilibration with atmospheric CO_2 . In order to determine whether changes in the salts resulting from drying might affect the kinetics of

* From the General Dynamics Corporation, Liquid Carbonics Division, San Carlos, Calif.

cleavage in egg populations, an experiment was performed in which eggs in normal sea water and in reconstituted water were fertilized simultaneously. The pH of the filtered sea water was 7.9, that of the (freshly) reconstituted sea water was 8.9. Cleavage was followed by the technique described above. The results are plotted in FIGURE 1. Clearly, neither the increased alkalinity of the reconstituted water, nor any changes in the salt composition resulting from the drying process, produce significant alterations in mean cleavage time or in the kinetics of cleavage for the population. Our experience has been, in

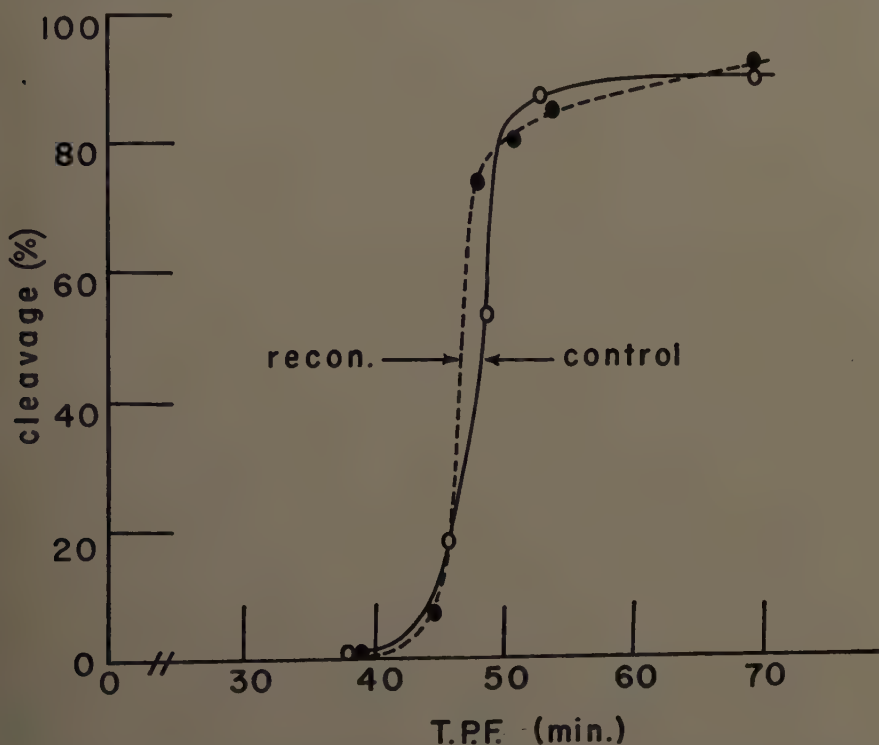


FIGURE 1. Time course of cleavages in two populations of *Arbacia* eggs. Control in normal filtered sea water, "recon" cells suspended in sea water reconstituted from dried salts and distilled water as described in the text. pH in control was 7.9, pH in "recon" was 8.9. T.P.F. = time postfertilization.

general, that slightly alkaline solutions tend to hasten, rather than to delay, cleavage. These findings are in accord with the experience of others. Thus, for example, Smith and Clowes¹⁹ have found that retardation of cleavage is observed for *Arbacia* eggs only at pH values below 6.0 and above 9.4.

In view of these earlier observations and of our own finding that the reconstitution procedure does not produce important changes in the salt composition of the medium, we have assumed in our further work that mitotic inhibition observed in D₂O-containing media is not a result of the small differences in acid-base properties between D₂O and H₂O. This conclusion has been reached by investigators of other biological effects of deuterium, for example, B. Cagianut.²⁰

Fertilization in heavy water. An experiment was performed whose aim was to determine the extent to which fertilization can be accomplished in solutions containing D_2O . To this end, samples of eggs were placed in a number of vessels in such a way that the concentration of cells was the same in each. The eggs had been tested earlier by inseminating a suspension in filtered sea water. The eggs in this batch showed more than 95 per cent fertilization, as evidenced by the elevation of normal fertilization membranes. In the actual experiment,

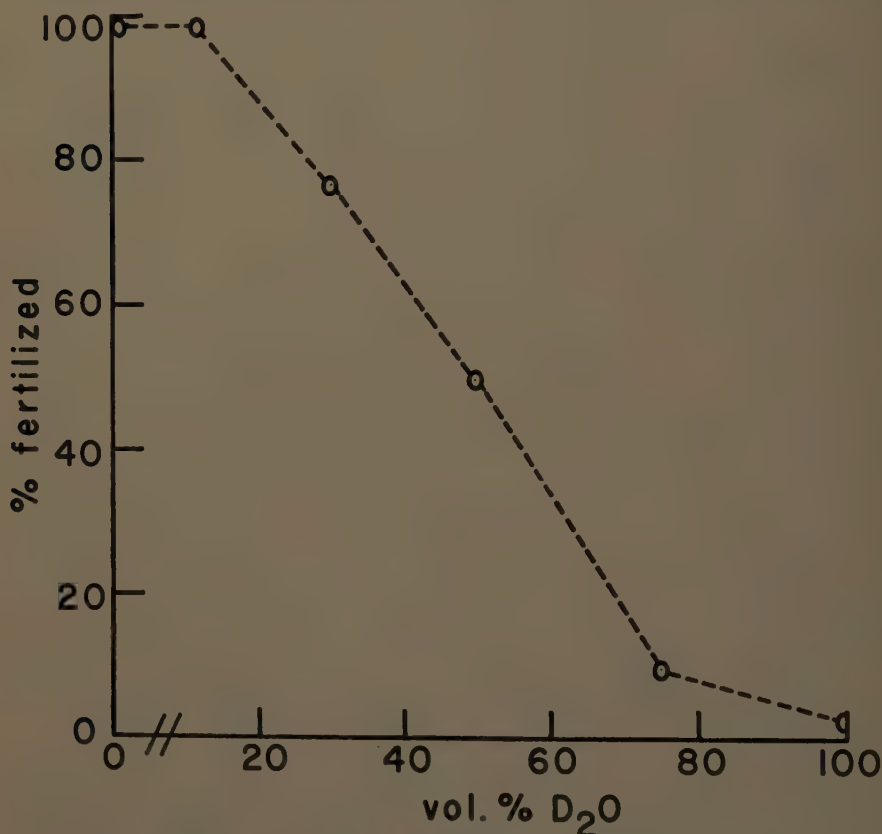


FIGURE 2. Fertilization in media containing various concentrations of D_2O . *Arbacia* eggs were counted as fertilized that had elevated a normal fertilization membrane within 5 min. after insemination of the egg suspension.

a second control sample was prepared from eggs suspended in reconstituted H_2O -sea water, and the remaining samples were in media with increasing D_2O content up to 99 per cent.

Preliminary examination of *Arbacia* sperm suspended in D_2O sea water revealed that sperm motility is not seriously affected for at least 10 min. after entry into the heavy water. This interval would normally be of sufficient length to assure fertilization of the eggs.

The results of the experiment are plotted in FIGURE 2. Those eggs were counted as fertilized that had elevated a fertilization membrane within five

min. after insemination. An examination 60 min. later showed that eggs that had not succeeded in elevating a membrane after 5 min. did not do so later, and did not divide.

Fertilization, or at least those processes involved in the elevation of the membrane, cannot take place in 99 per cent D₂O, and only in media whose D₂O content is less than 20 per cent does a normal percentage of the cells show that response. Since sperm motility seems little affected during the first few minutes at least after entry of the spermatozoa into D₂O, we conclude that the inhibitory action is upon the eggs themselves, perhaps simply upon the release and polymerization of the membrane precursors.

Cleavage: arrest and reversal. Fertilization is a phenomenon restricted to eggs, but the mitotic cycle is almost universal, and hence our decision was to bypass, for a time, the fertilization problem. This was accomplished by fertilizing all of the eggs for an experiment at the same time in normal sea water. Thereafter, when a specific time had elapsed, aliquots of the egg suspension were withdrawn and added to temperature-equilibrated reconstituted sea water or to D₂O-sea water. In this way, the effects of D₂O could be assessed at selected stages of the mitotic cycle upon the assumption that the deuterium in the medium equilibrates very rapidly with the hydrogen in the cell water. That such an assumption was justified is shown by the almost instantaneous effects to be described below. Also pertinent is the high permeability of the cell to water, and the fact that permeability to D₂O is the same as that to H₂O.¹⁰ Rapid exchange is also suggested by our inability to observe, after only a minute in D₂O, any volume changes of the kind predicted and observed by Brooks²¹ for other cells in D₂O, which means that osmotic differences between the medium and cell water had disappeared in less than a minute.

A suspension of eggs was fertilized under normal conditions and at 20° C. The eggs showed better than 95 per cent fertilization, and were kept in normal sea water with very gentle agitation for 15 min. At 20° most of the eggs were expected after 15 min. to be somewhat beyond the "monaster" stage, that is, pronuclei-fused, and astral rays spreading throughout the cytoplasm; this is preliminary to the division and migration of the centrosomes. Two aliquots were removed from the parent suspension; one was added to reconstituted sea water (H), and the other to D₂O-sea water (D). The final concentration of D₂O, after dilution by the normal water added with the eggs, was 96 per cent. All subsequent observations were made on three suspensions; H, D, and the normal sea water control (henceforth called C).

The results of this experiment and of the subsequent washing-out of the D₂O are shown in FIGURES 3 and 4. The counting data are plotted for three successive cleavage cycles in FIGURE 4. A few frames from the filmstrips of this experiment are reproduced in FIGURE 3.

The eggs began to cleave in C and in H at about 50 min. after fertilization. At that time, there were no divisions whatsoever in the D sample. During the time from 15 min. to furrowing, the samples were examined for the characteristic changes that accompany the mitotic cycle in *Arbacia* eggs. These changes took place normally in H as well as in C, but there were no changes in D. Indeed, during their entire stay in D₂O, these cells remained in the monaster stage.

In FIGURE 3, frame 12 shows the C eggs at 58 min. postfertilization, while frames 14 and 15 show H cells at 58.5 and 59 min., respectively. There are evidently no differences between the two control samples, since both show better than 95 per cent cleavage at this time. For comparison, the D cells are shown in frames 17, 22, and 23 at 63, 78, and 80 min., respectively. Frame 23 is at a higher magnification than used for counting, and it is included because it shows (1) that the "D" cells have been arrested at the monaster stage; and (2) that the peripheral cytoplasm has developed many small, clear areas. These

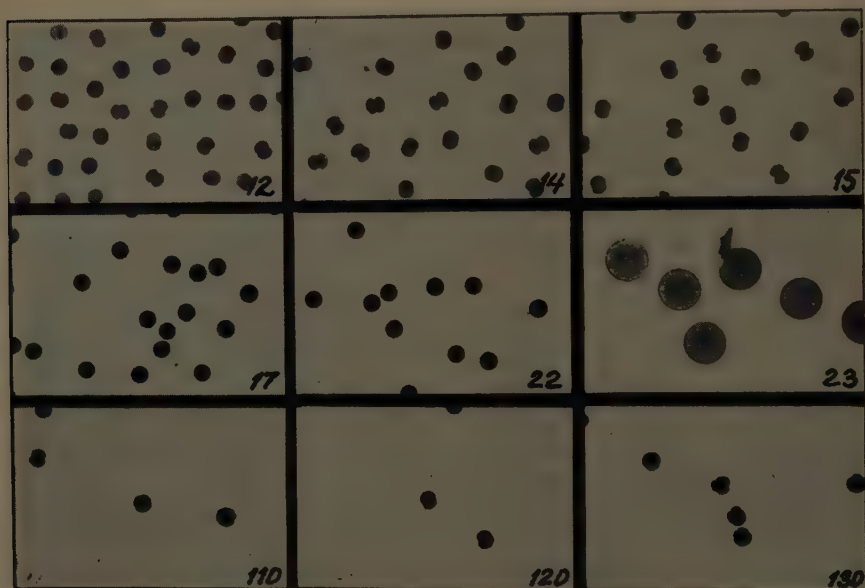


FIGURE 3. Arrest of mitosis and reversal of arrest. *Arbacia* eggs at 20° C. Eggs placed in D₂O 15 min. after fertilization. D₂O concentration: 96 per cent. Frame 12: eggs in normal sea water, 58 min. postfertilization. Frames 14 and 15: eggs in H₂O-reconstituted sea water at 58.5 and 59 min., respectively. Frames 17, 22, and 23: eggs in D₂O at 63, 78, and 80 min., respectively. Frame 23 shows arrest at monaster stage (15 min. postfertilization) and peripheral "pebbling" in cytoplasm. Frames 11D, 12D, and 13D: eggs washed free of D₂O, showing normal and irregular cleavages after reversal of mitotic arrest about 160 min. after fertilization and 60 min. after removal from D₂O.

latter are probably related to the pathological structures known as cytasters. It has been our experience that total blockade of mitosis such as is shown in these data continues, in concentrated D₂O, until the cells eventually cytolysis; that is, at D₂O concentrations greater than about 75 per cent there is no spontaneous escape from the mitotic arrest.

While the D cells continued blocked at the stage of entry into heavy water, the C and H populations continued to divide normally, and this is plotted for three successive cleavage cycles in FIGURE 4, which has been drawn semilogarithmically to accommodate the long period of observation. The open circles for the first normal cleavage represent counts of the C sample; the α marks are

counts of the H sample. These did not differ throughout the experiment in any way.

One hundred minutes after fertilization the D and H cells were removed (by centrifugation at low speed) from their media, washed several times, and resus-

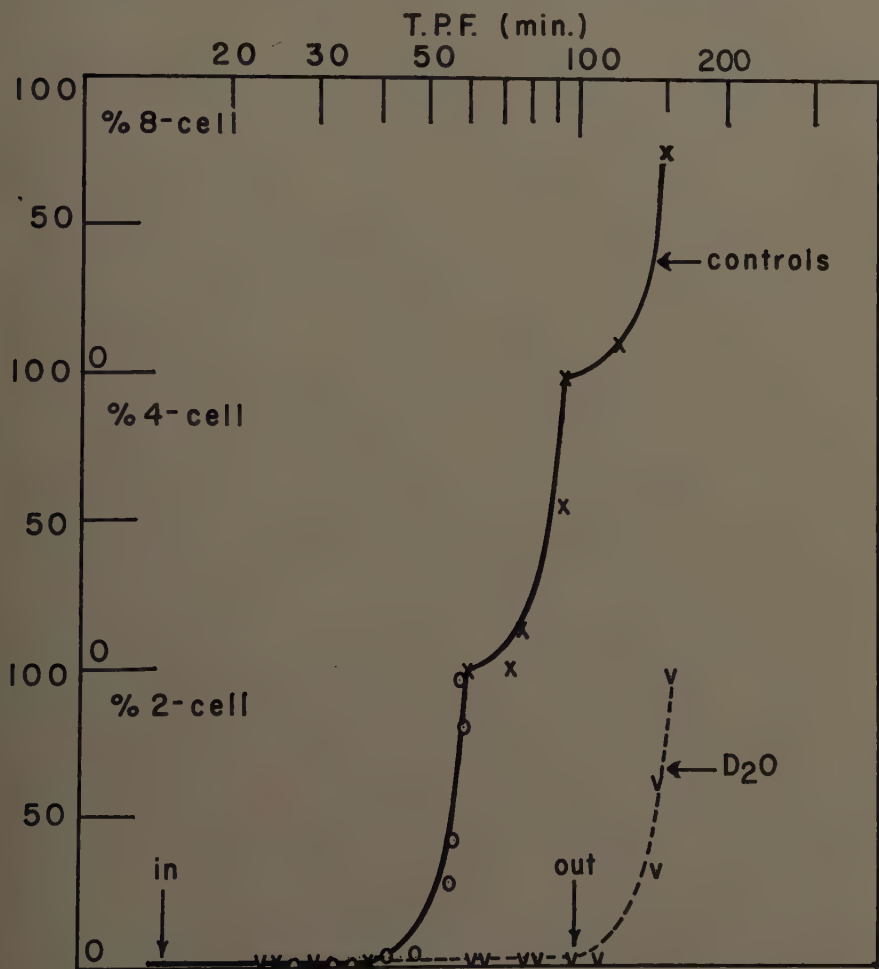


FIGURE 4. Counting data for experiment represented in FIGURE 3. Three division cycles in controls: open circles for cells in normal sea water, x for cells in H_2O -reconstituted sea water. $20^\circ C$. Experimentals (v) placed in 96 per cent D_2O at 15 min. postfertilization, washed free of D_2O at 100 min. postfertilization. Solid line shows percentages normal 2-, 4-, and 8-cell stages; dashed line refers to percentage of eggs showing at least one normal furrow.

pending in normal filtered sea water. This had, of course, no effect upon the H cells, which were normal anyway, but the D cells began to show cytological changes within 10 min. During the succeeding 30 min., most of the D cells showed "streaks," and then more or less abnormal "dumbbells"; they finally

divided, although in many cases into more than two blastomeres. Frames 11D, 12D, and 13D in FIGURE 3 show the types of division observed in the washed D samples at 160 min. postfertilization: normal 2-cell stages, normal 4-cell stages, irregular 2-cell stages, with 1 blastomere larger than the other, and irregular 3-, 5-, and 6-cell stages. In FIGURE 4 we have plotted simply the percentage of washed D cells showing at least one complete furrow. Evidently the mitotic block was released by the removal of the D_2O , and the kinetics of the resulting divisions are essentially normal for the population, although the divisions themselves were often not normal.

All of the samples, washed D, washed H, and C were allowed to remain on the sea-water table overnight. The following day, both C and washed H samples contained rapidly swimming embryos. In the washed D sample there were many (20 to 30 per cent of the original cell number) undifferentiated, multicellular masses, but the remainder of the cells had developed into normal-looking, swimming embryos not markedly delayed in their development with respect to the controls.

Cleavage inhibition by different concentrations of D_2O . A large volume of egg suspension was inseminated and kept on the water bath, with gentle agitation, for 20 min. The temperature was $22.5^\circ C$. Twenty min. after fertilization, most of the cells were in the "streak" stage, that is, the centrosomes were now separating and the mitotic spindle beginning to form, as a flattened fusiform body, between them and over the still-intact fusion nucleus. At this time, aliquots were removed from the parent suspension and added to temperature-equilibrated solutions in such a way that each had approximately the same cell density and the concentrations of D_2O were 0, 12.5, 25, 50, and 75 per cent.

Cleavage and development were followed as described above, and the data for the first two cleavage cycles in the controls are plotted in FIGURE 5. Curve A depicts the progress of the first two cleavages in the control (reconstituted sea water, in this case). Curve B refers to the suspension in 12.5 per cent D_2O . For this population, there has evidently been no alteration in the kinetics of division (actually, the cumulative probability in time for furrowing), but the excess deuterium has effected a simple delay in cleavage of 12 to 15 min. The curve marked C corresponds to the sample in 25 per cent D_2O . Here there has been a slight change in the shape of the cumulative probability curve, revealing an increased variance of cleavage times about the mean. This effect is enhanced during the second cleavage, which is not shown complete on the graph. The delay in first cleavage is here much larger than in the 12.5 per cent sample; indeed, the increment in the mean time to first cleavage is more than 75 per cent of the control time. Nevertheless, these eggs do continue to divide steadily, albeit slowly, so that the population as a whole progresses through 2-, 4-, and 8-cell stages and upward. Curve D is for the 50 per cent D_2O sample. Here there are profound changes in kinetics as well as in the cleavage time. About 25 per cent of the cells never divide at all; 50 per cent never enter the 4-cell stage. The remaining 50 per cent do divide, but very slowly and with great scatter of cleavage times. Curve E is for the eggs in 75 per cent D_2O . These cells are completely blocked for the first 4 or 5 hours,

after which most of the cells cytolize. One or 2 per cent of the cells show abnormal furrows.

These samples were allowed to incubate until both controls, that is, A and the parent suspension in filtered sea water, had produced rapidly swimming

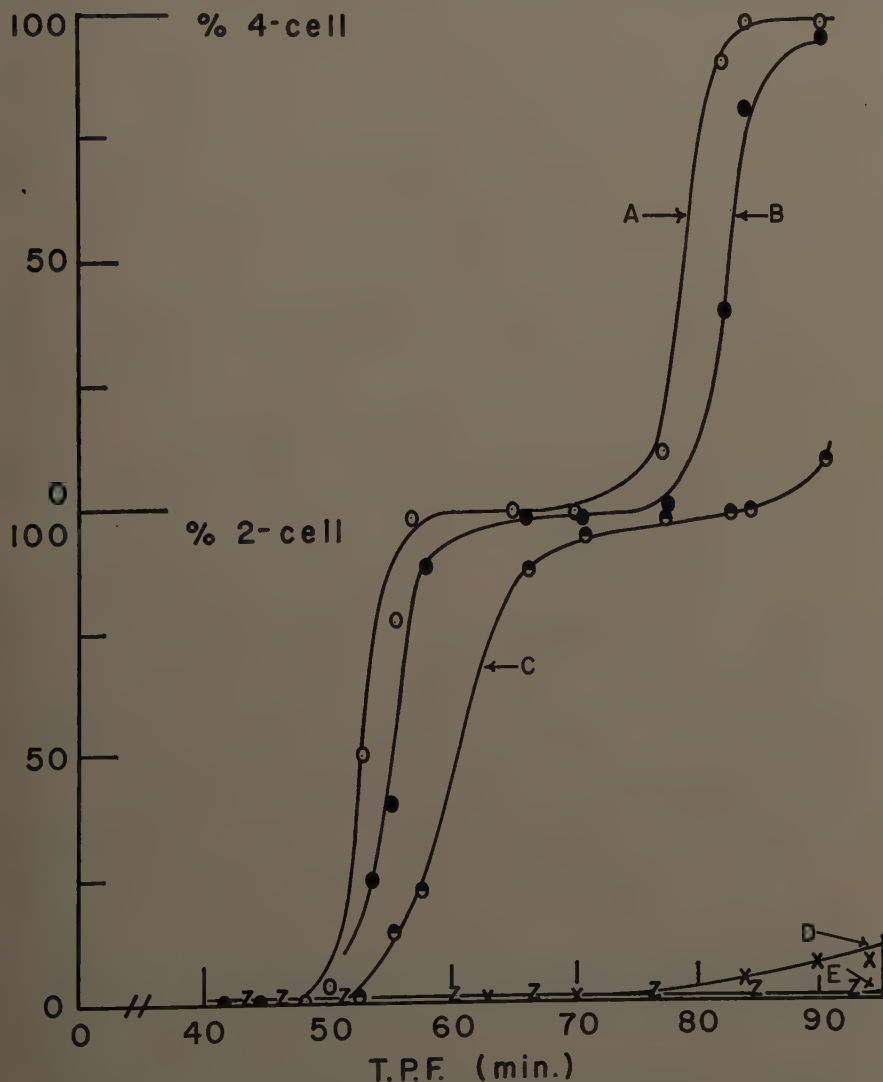


FIGURE 5. Kinetics of cleavages in different concentrations of D₂O. Eggs transferred from normal sea water to D₂O-enriched media at 20 min. after fertilization. Temperature 22.5° C. *Arbacia* eggs. Ordinates: percentages of normal 2- and 4-cell stages. Abcissa: time postfertilization. Curve A: reconstituted H₂O-sea water (no D₂O enrichment). Curve B: 12.5 per cent D₂O. Curve C: 25 per cent D₂O. Curve D: 50 per cent D₂O. Curve E: 75 per cent D₂O. Open circles correspond to A, filled circles to B, half-filled circles to C, X to D, and Z to E.

larvae. The others were then examined, with the following results. In B (12.5 per cent D_2O), there were swimming larvae indistinguishable from those in A; in C, the same was true, although closer examination of fixed material might have revealed some lag in development; in D (50 per cent D_2O) there were 25 per cent 2-cell stages, 25 per cent undivided, cytolized eggs, and the remaining 50 per cent had divided many times but had neither differentiated visibly nor hatched to produce free-swimming forms. In E (75 per cent D_2O), there had been a few irregular divisions in the uncyclized eggs, but most of the cells were dead and had never divided.

Examination of the kinetics of cleavage in these cells thus provides evidence of the considerable sensitivity of the mitotic process to quite low concentrations of deuterium. Below 50 per cent, the effect is essentially a simple delay; above 50 per cent, the cleavage time begins to scatter very widely about the mean and many cells never divide; above 75 per cent, mitotic block is practically complete for the population.

Inhibition of late stages and stabilization of the spindle. A number of experiments were performed in which fertilized eggs were immersed in D_2O at successively longer intervals after fertilization. When the D_2O concentrations were sufficiently high, the results were quite striking; indeed, it is in this regard that D_2O demonstrates its most unusual properties as an antimitotic agent. There appeared to be no stage of mitosis with particular sensitivity: all stages were blocked, and reversibly so, including even furrowing. These experiments were repeated with *Chaetopterus* eggs with similar results.

Of interest to us was the fact that mitosis could be stopped even after metaphase since, although other antimitotics can do the same, it is at the expense of destruction of the spindle or furrow regression. The D_2O effect appeared to be different: whereas cleavage could be stopped at any stage, late blockade did not cause appreciable furrow regression but merely an arrest of the process of furrowing. Likewise, arrest after metaphase did not cause regression of the spindle in so far, at least, as the clearly visible, mature dumbbell stage in the living cell represents an intact achromatic figure. Late arrest, after metaphase and before cytokinesis, appeared instead to stabilize the spindle.

In order to examine this stabilization more closely, a suspension of cells was inseminated in the usual way and incubated at $25^\circ C$. for 40 min. At this time, most of the cells had surely entered anaphase. Certainly, all of the cells showed easily-identified achromatic figures, and a small number had already begun to furrow. At this time, aliquots were removed from the parent suspension and added to H and D media, the final concentration of D_2O being, in this case, 86 per cent. The results of the counting are shown in FIGURE 6. Whereas the controls (H) cleaved normally about 5 min. after sampling (45 min. post fertilization), only 5 to 7 per cent of the eggs in D cleaved, the rest remaining in the condition seen at the time of immersion in D_2O .

The D cells (as well as the controls) were photographed at 1 or 2 min. intervals from the time of sampling until 57 min. after fertilization. Later analysis of the filmstrips showed that the anaphase spindles (actually dumbbell figures) had remained intact in all of the D cells. The photographs for this experiment were made on direct positive film, and it is most instructive to project alternate

frames from the control and from D ; the impression is that the cells in D_2O have been frozen into immobility.

Seventeen min. after their addition to H and D , the cells from these suspensions were washed and resuspended in normal sea water. At 69 min. after fertilization, 85 per cent of the D cells had divided and, because of their relatively brief stay in D_2O (17 min.), these divisions were mostly normal cleavages into 2 equal-sized blastomeres. These findings are summarized in the graph in

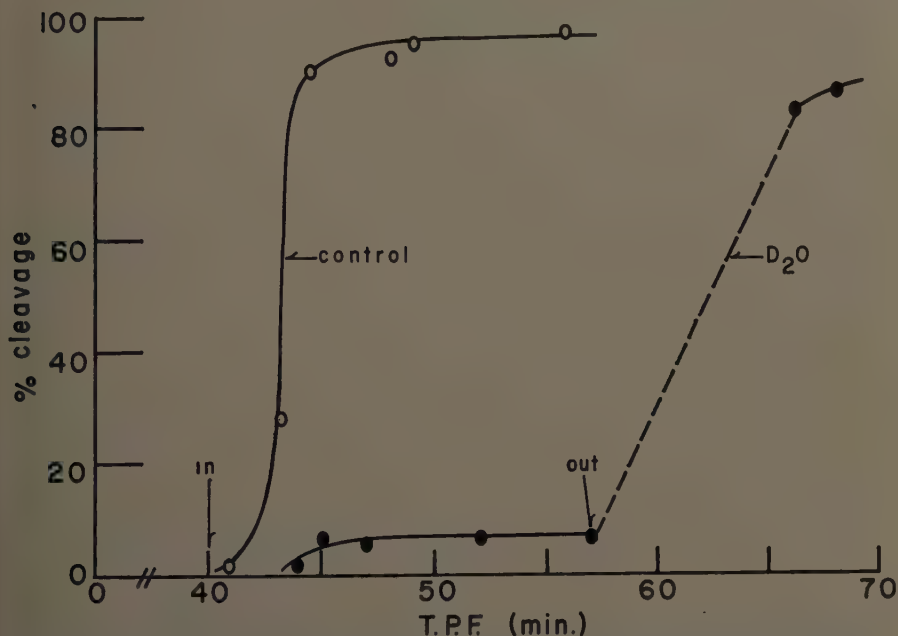


FIGURE 6. Antimitotic action of D_2O very late in the mitotic cycle. *Arbacia* eggs incubated at $25^\circ C$. for 40 min. Aliquots then removed and placed in H_2O -reconstituted sea water (curve labeled control . . . open circles) and in 86 per cent D_2O -sea water (curve labeled D_2O . . . filled circles). Ordinates: percentages normal 2-cell stages. Abscissa: time post-fertilization. Dashed portion of D_2O curve represents period during which no counts could be made because of handling subsequent to removal of these cells to normal water (begun at "out").

FIGURE 6. The dashed segment of the D_2O curve has been distinguished from the rest of the line because we were unable to make records in that interval.

It is difficult to study the effect of immersion in D_2O beyond the time when about 10 per cent of the population show furrows; this is because in a suspension of normal cells, the interval between 10 per cent furrows and 80 per cent furrows is very short, often no more than 2 or 3 min. It is very difficult to make transfers and counts at this time. We have nevertheless made such transfers as late as the time when 30 per cent of the cells had furrowed. When this was done, it was found that whereas the controls completed the first cleavage within 2 or 3 min., only 40 per cent of the D_2O (96 per cent) cells showed furrows 30 min. later. When these cells were returned to sea water, they promptly

completed the first division cycle; interestingly enough, however, many of the divisions were now multipolar.

Cytoplasmic ultrastructure. A suspension of fertilized eggs was held at 25° C. for 26 min., whereupon 2 aliquots were removed and added, respectively, to H and D (95 per cent) media. At the time of transfer, the cells were in the streak stage. All three samples, C, H, and D, were observed at intervals, and it was clear that, whereas the cytologic changes of the mitotic cycle were progressing normally in C and H, the D cells had been arrested at the stage of their entry into D₂O.

Forty-six min. after fertilization, when controls were presumed to be in late anaphase or telophase, the H and D cells were collected quickly by centrifugation and fixed in buffered 1 per cent OsO₄ solution. Subsequent preparation and electron microscopy were carried out as described by Gross *et al.*¹⁸ In all cases where the plane of section was favorable for identification of the stage of mitosis, the controls were seen to have been fixed in late anaphase or in early telophase, but the D cells were in the streak stage, with the nuclear membrane intact. Since the ultrastructural features of normal cells have been described elsewhere in detail,^{18,22} the discussion to follow will deal only with differences between D cells and controls.

Superficially, the D cells looked like normal cells in the streak stage. They showed an intact nucleus with two conical regions, representing the forming mitotic spindle, radiating from opposite nuclear poles. The conical regions are easily seen because they are regions of gelation from which, in the *Arbacia* egg, the abundant large cytoplasmic inclusions are extruded. Normally, of course, this structure should have given way, 15 min. prior to fixation, to the metaphase spindle. The cytoplasmic particulates in the deuterated eggs appeared normal, that is, yolk particles, pigment spaces, mitochondria, annulate lamellae, and ribosomes did not differ from those seen in the controls, but, as will be seen, their distribution in the ground cytoplasm may have been altered.

FIGURE 7 is a low magnification survey micrograph of one of the control cells, showing the region of the mitotic spindle. Characteristically, the larger inclusion bodies have been excluded from the spindle region, whose borders they therefore delimit. A few yolk particles and pigment vesicle spaces are seen at lower left and upper right, and the long axis of the spindle approximates the upper left-lower right diagonal. The two groups of chromosomes have become highly vesiculate, a sign of their approaching fusion (telophase), and the appearance of this cell is quite characteristic for late anaphase in *Arbacia* eggs.

FIGURE 8, at the same magnification, shows the condition of the D cells at the time of fixation. The still-intact nuclear membrane is seen at the upper right, with the axis of the presumptive spindle, the streak, running from upper right to lower left. This electron micrograph also shows the extruded larger particulates, mainly yolk and mitochondria, in the cytoplasm surrounding the streak. A few profiles of annulate lamellae are seen within the streak. The 15- μ ribosomes are scattered profusely throughout the ground cytoplasm, outside and inside the streak. Both ribosomes and annulate lamellae seem more concentrated within the streak than without, but this is a condition observed also in normal cells.

Aside from the obvious blockade at, without destruction of, the streak, there

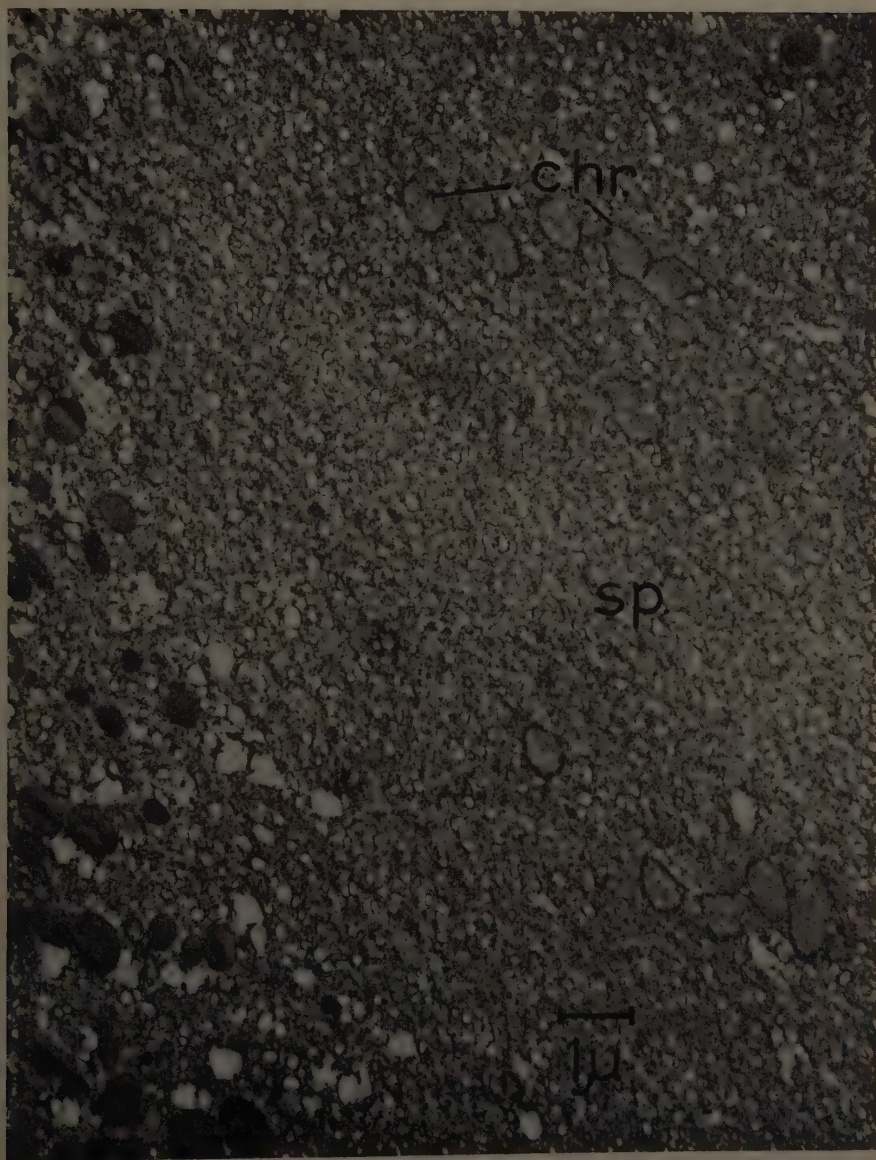


FIGURE 7. Electron micrograph of *Arbacia* egg kept in normal sea water at 25° C. for 26 min., transferred to H₂O-reconstituted sea water, then fixed at 46 to 48 min. after fertilization. Fixation: buffered 1 per cent OsO₄. ×9780. This is a control cell, sectioned approximately through the long axis of the anaphase figure. A few yolk particles and pigment spaces at lower left delimit the achromatic figure. The spindle is labeled sp., and one of the two groups of vesiculating chromosomes is at "chr."

is only one ultrastructural difference between D_2O blocked cells and normal cells at the same stage of mitosis. The larger particulates are distributed homogeneously in normal cells, whereas in the D_2O -blocked cells, these bodies show a tendency to gather into clumps, leaving spheres of ground cytoplasm of varying diameter free of such particles. These clear regions resemble the spindle

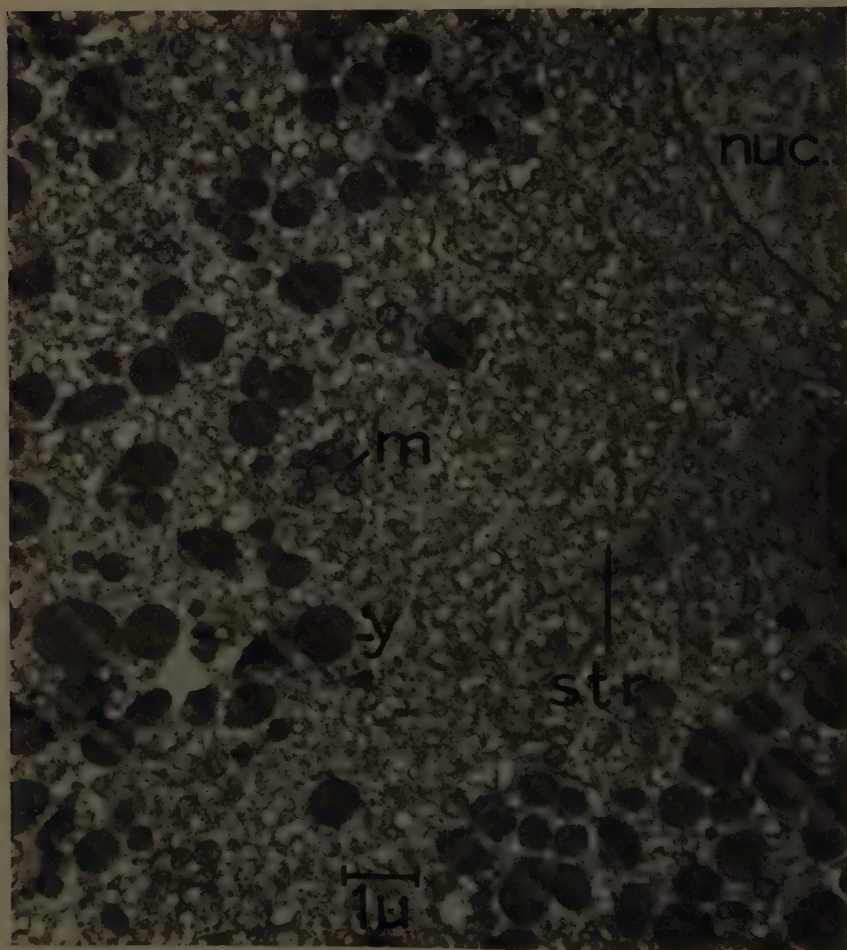


FIGURE 8. Electron micrograph of *Arbacia* egg kept in normal sea water at 25° C. for 26 min., then transferred to 95 per cent D_2O -sea water, and fixed at 46 to 48 min. after fertilization. Fixation: buffered 1 per cent OsO_4 . $\times 9780$. This micrograph is to be compared with that for the control in FIGURE 7. The cell is still in the streak stage, that is, the same stage of mitosis as that in which the cell entered the D_2O solution at 26 min. postfertilization. The nuclear membrane and nucleus are at upper right, and the clear region of the streak (str.) is delimited by the particles on both sides. The axis of the streak runs approximately upper right to lower left. Mitochondria (m) and yolk particles (y) are of normal morphology. These particulates show a tendency to clump and are therefore distributed less homogeneously than in a normal cell at the same stage of mitosis. See the text for discussion.

itself, for with osmium fixation, the spindles, too, contain mainly ribosomes and vesicles.²² We interpret these clear spaces as "islands" of gelation, on the basis that gelled regions of the normal egg expel their yolk, pigment, and mitochondria. Such an interpretation is in accord with light microscopy and with the "viscosity" measurements to be described below.

As to the appearance of blocked eggs under the light microscope: when these have been resident in concentrated D₂O for more than 15 min., the peripheral cytoplasm assumes a curious "pebbled" character (compare FIGURE 3, frame 23), which is quickly reversed upon removal to normal water. The "pebbling" reaction is reminiscent of cytaster formation after treatment of eggs with certain parthenogenetic chemicals. It is doubtful that the "pebbling" seen in the deuterated eggs represents the formation of true cytasters, since the clear spaces vary greatly in size and there can be a very great number of them. It is not unreasonable, however, to suppose that the pebbling results from a similar condensation, or gelation, in many places in the ground cytoplasm.

Chaetopterus eggs and "viscosity" measurements. The effect of D₂O on eggs of *Chaetopterus* is in most respects like that on *Arbacia* eggs. Differences in response are quantitative only; the *Chaetopterus* eggs are even more sensitive to D₂O than are the *Arbacia* eggs. The increased sensitivity is manifested by greater antimitotic effectiveness of D₂O at concentrations below 70 per cent, by greater difficulty in reversing the arrest, by a tendency of the cells to shrink after approximately 30 min. in D₂O, and by greatly increased numbers of abnormal and multipolar cleavages after successful reversal of mitotic arrest.

Because of the high antimitotic efficiency of D₂O in this cell and because the stratification behavior of its cytoplasmic particles has been studied thoroughly by Heilbrunn and Wilson,²³ a number of observations were made upon *unfertilized Chaetopterus* eggs. The control eggs were kept, after release from the parapodia and several washings in sea water, in reconstituted H₂O-sea water. The experimental cells were immersed at the same time in 60% D₂O, a concentration sufficiently high to produce complete mitotic arrest in this cell.

The cells were centrifuged in a hand-operated machine, at a relative centrifugal force of 2200 g. The end point selected was "zoning," that is, formation of a clearly defined centripetal "cap" of oil droplets and a similarly well-defined clear zone below. Such zoning is a signal of the incipient stratification of larger cytoplasmic inclusions, and the speed of zoning is related indirectly to a "viscosity" of the cytoplasm. Exact interpretation of stratification times in terms of viscosity is difficult, and it requires information beyond that obtainable from an experiment performed at a single force, including data on shear dependence at low shear rates and the flow pattern about the moving particles. We made no attempt to calculate such viscosities, but considered simply that the time required to produce the end point is proportional to a deformation resistance in the cytoplasm that may in turn be defined as a function of the viscosity. An increase in the time then becomes a function of an increased relative viscosity, deuterated to control eggs, at the shear stress imposed by centrifugation. Such an increase in deformation resistance would normally indicate that interactions between cytoplasmic macromolecules and particles had become stronger, or that there were more interaction sites than in the control.

The former is a more likely interpretation in view of the nature of the treatment.

Under the conditions selected, the mean stratification time for the control eggs was 10 sec. For the cells in 60 per cent D_2O , the mean stratification time was more than 60 sec. It is important to note that these measurements were made before any cytological changes (such as the pebbling in *Arbacia* eggs) could be detected, and that the diameters of the treated eggs were identical before, during, and after the measurements, with those of the controls.

Many measurements were made on both *Arbacia* and *Chaetopterus* eggs in various concentrations of D_2O , from photographs, and directly under the microscope, with a filar micrometer. If D_2O , because of its lower vapor pressure, were hypertonic to normal water, then transient shrinkage would be observed, and Brooks²¹ has gathered some evidence in favor of such an idea. In these cells, however, we could detect no changes in volume at the first observation, which was usually about 30 sec. after immersion in the D_2O ; nor were there volume changes with respect to the controls until long periods had passed (that is, hours in *Arbacia*). The late shrinkage was associated, however, with cytolysis. Hence exchanges between cell water and D_2O were very fast; consequently the elevation of cytoplasmic rigidity in D_2O is not a result of hypertonicity of the medium. It may also be noted that if the viscosity increment were due to the D_2O/H_2O viscosity difference, the stratification times could have been increased at most by 25 per cent (that is, for pure D_2O , not 60 per cent). An increase of 600 per cent in stratification time, for cells in 60 per cent D_2O , would therefore suggest changes in the interactions among cytoplasmic macromolecules.

Discussion

The following are the more interesting aspects of the antimitotic action of deuterium oxide:

(1) The block to mitosis occurs at any stage of the process since, in all cases, eggs immersed in concentrated (75 per cent or more) D_2O -sea waters remain cytologically unchanged, regardless of the stage of mitosis obtaining at the time of immersion. This is true even for the late, postanaphase events of mitosis and, to a significant extent, for cytokinesis. Deuterium thus exhibits an unusual catholicity of action against the formation and operation of mitotic spindles, asters, and cleavage furrows. These properties may not necessarily be unique, but they segregate D_2O from the vast majority of chemical inhibitors of cell division.

(2) Whereas other agents, such as the urethanes,²⁴ which are capable of interfering with the late stages of mitosis, seem to do so by destroying or dissolving the spindle, or by causing regression of cleavage furrows, D_2O appears to act by "freezing" or stabilizing these structures so that they cannot complete normal growth or normal movement.

(3) This stabilization of the achromatic figure and of the furrows is doubtless related to the fact that relatively low concentrations of D_2O produce a sharp increase in the viscosity of the cytoplasm in nondividing cells, the viscosity being measured as a stratification time.

Without more data than are now available, it is not possible to propose with

any certainty a mechanism by which D₂O exerts these effects. It is possible, however, even at this early stage of the work, to outline an hypothesis whose success or failure may, as a kind of bonus, help in assessing the types of chemical bonds and intermolecular forces that are involved in the maintenance of such ordered assemblies as the mitotic spindle.

How, to begin, does D₂O differ from "light" water? Kirshenbaum²⁵ has listed the physical properties of the two liquids, and many of the differences seem to suggest differences in the strengths of the hydrogen bonds. Thus D₂O melts at 3.79° C. instead of at zero; its vapor pressure at 20° C. is 15.25 mm. Hg instead of 17.54; its heat of fusion is 1515 cal./mole instead of 1436; its viscosity is about 25 per cent higher than that of H₂O, at low temperatures. Other properties of interest here are the ionization constants: 1.9×10^{-15} for D₂O ($K_w = 1 \times 10^{-14}$), and the densities: 1.10775 gm./ml. for D₂O ($d_w = 0.99707$) at 20° C.

The differences in vapor pressure, density, viscosity, and ionization, while appreciable, do not appear to account, on the basis of the data given above, for the drastic antimitotic action of D₂O. Most investigators who have studied the biological effects of deuterium have assumed that the basis for such effects must, instead, be in the primary and secondary isotope effects upon reaction rates for protium and deuterium reactants, respectively. This assumption includes the further assumption that critical substrates and enzymes exchange their hydrogen for deuterium relatively quickly once the organisms are exposed to a deuterated medium. This accessory assumption is certainly justified if the hydrogens concerned are those in hydroxyl, amide, and carboxyl functions; it may be less justified for hydrogens bonded to carbon and for certain very slowly exchangeable hydrogens found in macromolecules.²⁶

It is certainly true that bonds to D will be broken less readily than those to H, because the activation energy for such breakage will be somewhat larger in the case of the D compound. This results from a lower zero-point energy, among other things. The difference arises from the large mass difference between the isotopes, since the zero-point energy depends upon an equilibrium vibration frequency and hence upon the reduced mass of the molecule. Katz *et al.*³ give such maximal values for the isotope effect at 25° C. that a C—H bond may be cleaved 7 times faster than a C—D bond, a =N—H bond 8.5 times faster than a =N—D bond, and an O—H bond 10.6 times faster than an O—D bond.

This isotope effect has been extensively investigated, and many examples are given in Wiberg's review.⁸ The primary isotope effect usually results in a decrease in reaction velocity of a few to several hundred per cent, depending upon the reactants. Unusual cases are encountered, especially among secondary effects, where the rate is increased by deuteration. Enzyme-catalyzed reactions show isotope effects similar to those observed in ordinary organic processes, although in the *in vitro* biological systems, the effects often tend to be small. To choose but one of many possible examples, the oxidation of methyl blue in the presence of alcohol dehydrogenase is unchanged when 99.6 per cent D₂O is substituted for normal water, whereas the same authors (Shibata and Watanabe²⁷) show a reduction in rate of 64 to 86 per cent for the breakdown of D₂O₂ (instead of H₂O₂) by catalase in 99.6 per cent D₂O. The net effect in

biological systems seems, in any case, to be in the direction of a depression of general metabolic activity.^{3,9} There may also be certain alterations in the pattern of metabolic pathways, as shown in the data of Moses *et al.*⁶ Such changes in pattern are not unexpected if the several reactions of a multienzyme sequence can suffer different rate changes. The metabolic effect can thus be large enough, although the fact of adaptation of microorganisms to growth in pure D₂O⁹ suggests that they are not necessarily inconsistent with life. What must now be asked is this: Can the antimitotic action described above be attributed to an isotope effect upon the rates of metabolic processes?

To approach an answer to such a question, it should be noted that deuterium affects the cells of higher organisms more drastically than it does microorganisms. Although D₂O does depress the growth of microorganisms, at least at higher concentrations, they can fare quite well in media containing 30 to 50 per cent D₂O. Beyond these levels, unadapted organisms show an inhibition of cell division (for example, cell enlargement) with less severe effects upon metabolism. Adapted organisms, however, grow reasonably well in 99+ per cent D₂O.⁹

The situation in higher organisms is different. D₂O levels of 35 per cent in the body water, in rats and mice, produce severe systemic toxicity, with weakness, neuromuscular hyperexcitability, bradycardia, and stupor, culminating in death.³ Somewhat lower concentrations reduce markedly the reproductive potential of the animals,^{7,9} surely in part by a reduction in gametogenesis. Interference with tumor growth has been demonstrated.³ Inhibition of mitosis in invertebrate eggs has been obtained with quite low concentrations of D₂O.¹⁶ There are, unfortunately, insufficient recent data concerning mitotic inhibition in higher plants, although one of the first biological experiments was that of Lewis,² who showed complete inhibition of macrosporic development in tobacco seeds immersed in D₂O. Crumley and Meyer²⁸ have shown more recently that retardation of germination rate for a number of seed plants is an increasing function of the concentration of D₂O. Finally, the cleavage of frog eggs is affected much as is the division of marine invertebrate eggs, as was shown by Ussing,¹¹ and mitosis in cultured cells of mammalian origin is likewise strongly inhibited.^{9,20}

To us these findings suggest that D₂O plays havoc more readily, and at lower concentrations, with cell division than with metabolism and the synthesis of cellular materials. The ability of certain microorganisms to adapt to division in D₂O may be a result of the fact that they can accomplish partitioning of the genetic material and fission of the cell without the formation and operation of a large and complex achromatic apparatus.

These observations make less attractive at first glance the hypothesis that D₂O inhibits mitosis by a reduction of metabolism. However, perhaps the antimitotic effect is exerted upon a metabolic system, or even upon a single chemical reaction, which is critical and must be maintained throughout the whole process of cell division; or is the effect, rather, upon some physical or structural property peculiar to the dividing cell?

It has always been difficult to reconcile the magnitudes of the chemical and biological deuterium isotope effects. One proposal, just discussed, is that small changes in the rates of individual reactions can interact in such a way as to pro-

duce large depressions of biological activity. Such an explanation, however, does not satisfactorily account for the great differences in sensitivity to deuterium of cell division and cell metabolism (and growth), respectively.

One alternative, suggested to account for the antimitotic effect, is actually a form of the "critical pathway" or "critical reaction" hypothesis. This is to assume that certain macromolecules, whose synthesis or stability is essential for cell division, cannot properly be synthesized or retain the required stability in high concentrations of D₂O. Protein and nucleic acids are, of course, obvious candidates, but since cell growth does continue, albeit to a reduced extent, even in 99+ per cent D₂O, one must assume that protein and RNA are being made, and hence the most likely candidate for the "critical species" would be DNA. Now Moses *et al.*⁶ assumed, and Calvin *et al.*²⁹ have demonstrated, that the strength of deuterium bonds differs, in polymer-solvent systems, from that of hydrogen bonds and, although they have not ventured a statement about the direction of the change, the suggestion⁶ for biological systems is that the normal structure of macromolecules such as DNA, which is maintained by extensive intramolecular hydrogen bonding, cannot be properly maintained when hydrogens are substituted to a significant extent by deuterium. This is in many ways a reasonable proposal, particularly because it permits a cooperative effect of many bonds that would greatly increase the magnitude of the over-all isotope effect. This is in the direction required by the biological data.

To use a heuristic model, let the probability of breaking one H-bond, $p < 1$, be changed a few per cent by isotopic substitution. If a reaction required breakage of one bond, then the rate expression would be a function of p . However, assume that for a reaction involving a macromolecule, n bonds must be broken simultaneously. For the hydrogenated macromolecule, the probability function would contain an expression ($P_{\text{tot}} = p^n$). For the deuterated macromolecule, where p has been changed to $(p + q)$, q being plus or minus a few per cent of p , the probability expression would contain ($P_{\text{tot}} = [p + q]^n$), which would, of course, produce a very great change in the over-all rate for large n .

It is quite possible that effects of this type do influence the stability of nucleic acids in deuterated cells, but can they account for the antimitotic effect? The answer to this must be no, since mitosis is blocked at stages later than DNA duplication, and later even than the separation of the chromatids. Thus, whether deuteration affects the configurational stability of RNA, DNA, or proteins, *in vivo*, and whether D₂O turns out to be a mutagen (as such a proposal implies), it is clear that the blockade of mitosis described above depends upon a different mechanism (it may be that the less severe antidivision effects observed in some microorganisms do arise from such a mechanism, however).

As already suggested, however, it is difficult to attribute the antimitotic effect solely to interference with metabolism, for the following reasons:

(1) Such interference seems to become important in microorganisms and in other metabolizing systems only at D₂O concentrations much higher than those required to block mitosis.

(2) The antimitotic effect is exhibited almost immediately upon immersion of the dividing cells in D₂O, as though the rate-limiting step for mitotic block were no more than the exchange of cell water for D₂O. Such speed would not be expected if the effect were a cumulative depression of metabolism.

(3) The antimitotic effect is manifested at all points in the mitotic cycle, and certainly well after metaphase has been completed. As Swann⁸⁰ has shown, however, the mitotic cycle becomes independent of ordinary energy sources once the "energy reservoir" is filled, just prior to the assembly of the achromatic figure. Inhibitors of oxidative metabolism, for example, exert their effects on mitosis before, but not after, the appearance of the fully-developed achromatic figure. Such agents, when applied during the later stages of the cycle, inhibit the next division, but not the current one. Since D_2O acts after the energy reservoir has been filled, its effects cannot be attributed simply to changes in the rates of the reactions in the energy-yielding pathways.

There remains the possibility that some critical single reaction, unique to and necessary for mitosis, and required at every instant during the entire cycle, including cytokinesis, is specifically blocked even in low concentrations of D_2O . However, we know of no such chemical reaction, and it is difficult at present to imagine one, so that this possibility may, at least for the moment, be set aside.

There remains the possibility that D_2O acts directly upon the mitotic apparatus, forming or intact, and upon whatever other organized structures are involved in the growth of the cleavage furrow. Such a proposal is supported by the remarkable stabilization of these structures in the presence of D_2O . If heavy water were able to "freeze" the mitotic apparatus, either during the assembly or during the operation thereof, and if it were similarly able to "harden" the cell cortex in such a way as to impede furrowing, then the molecular mechanism of such freezing would be the mechanism of mitotic inhibition, since it is indeed stabilization, and not destruction of these elements, that is observed. Significant in this regard is the finding that even at concentrations as low as 60 per cent, D_2O elicits a sixfold increase in the viscosity of the cytoplasm. Since this viscosity may well be anomalous in the sense of increased values at very low shear rates, it is not unreasonable to suppose that the assembly and operation of microscopic structures requiring the breakage and formation of many hydrogen bonds would be impeded.

It is here that the cooperative effect can be transferred from the hypothesis stated above in connection with macromolecular stability. If there is a difference in the strength of D bonds compared to H bonds, as the properties of D_2O and H_2O suggest, and if such differences can be magnified by cooperative effects in systems where many hydrogen bonds give a structure its three-dimensional order, then the extensive substitution of D for H in the hydrogen bonding sites ought seriously to affect its operation. If the D bonds are weaker in such a system, then the structure should suffer a loss in stability; if the D bonds are stronger, as we believe they may be, then the structure would be stabilized and made more rigid, preventing normal changes in form.

It is proposed, therefore, as a working hypothesis, that one important mechanism for the antimitotic effect of D_2O is an increased rigidity of macromolecular superlattices in the cytoplasm, especially an immobilization of the mitotic apparatus at all stages of its formation and operation. This hypothesis has two consequences. First, it ascribes great importance to hydrogen bonds in the maintenance of the orderly structure of the achromatic figure and indeed, of the ground cytoplasm as a whole. It remains a possibility that the effect

is produced by conversion of —SH groups to —SD groups, but although this possibility cannot at present be discounted, it is less in accord with the data, particularly in view of Mazia's¹⁴ lucid accounts of the problem, in which it is made evident that —S—S— cross linkages do not alone account for the structural integrity of the entire mitotic apparatus. It may well be that hydrogen bonds and salt linkages play an equally important role.

A second consequence of the hypothesis is that substitution of D for H in some polymer-solvent systems will increase the strength of intermolecular cross links when these are hydrogen bonds, producing larger aggregates and higher viscosity or increased gel rigidity. A biological test of this proposal has been made, that is, the measurement of stratification time for inclusion bodies in deuterated and normal cells. The results support the hypothesis. Another approach is via the study of chemical models *in vitro*. Thus far, only the results of Calvin *et al.*²⁹ have been published, and these are not directly applicable, although they can be interpreted as indicating that N···D—O bonds in a helical polypeptide chain stabilize the structure more readily than do the ordinary N···H—O bonds. Such a result is suggestive, but does not aid directly in assessing the relative strengths of D and H bonds between polymer molecules in a given solvent. We have, however, begun a separate study of this question, and preliminary results obtained with gelatin solutions and gels suggest that, whereas the shape parameters of the gelatin "molecule" are not greatly altered in D₂O, the intermolecular cross-links, at finite concentrations, increase either in strength or in number. The cross-linkages are probably mainly hydrogen bonds. These data are to be published elsewhere.

Whether or not the model experiments ultimately support the hypothesis, it will be important to continue its testing in the living cell, for even a partial solution of the problem of mitotic blockade by isotopic substitution should yield useful information about the molecular events underlying cell division.

Summary

At concentrations higher than about 75 per cent, deuterium oxide blocks cell division in the eggs of the sea urchin *Arbacia punctulata* and in those of the annelid *Chaetopterus pergamentaceus*. The block is reversible, although cells that have been kept in a deuterium-rich medium for long periods relative to the time for one mitotic cycle may show multipolar and irregular divisions after removal to normal water, and may subsequently cytolyze. The anti-mitotic action is manifested at all stages of the mitotic cycle and, to a significant extent, during cytokinesis.

An important feature of the mitotic block is that it becomes effective almost immediately after the cells are placed in D₂O and is quickly reversed when the cells are washed free of D₂O.

Whereas D₂O blocks mitosis at all stages it does not destroy the achromatic apparatus, nor does it cause wholesale regression of cleavage furrows. Instead, it exerts a powerful stabilizing effect upon these structures. This appears to be related to a sharp and reversible increase in cytoplasmic viscosity evoked by immersion of nondividing cells in D₂O.

Examination of a number of alternative explanations of the effect leaves as the most useful working hypothesis a rigidification of the mitotic apparatus,

possibly through a small increase in the strength of individual hydrogen bonds which cross-link macromolecules or small particles. For large, multiply cross-linked structures, such changes for individual bonds would produce very large cooperative effects in the direction required by the data.

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REGENERATION AND CELL DIVISION IN TWO PROTOZOAN SPECIES*

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The effects of daily amputation of a mass of protozoan cytoplasm have led to somewhat conflicting results.^{1,7,14} However, the concept has arisen from these studies that an organism could be kept from dividing, perhaps indefinitely, by daily amputations of a critical quantity of cytoplasm. The removal of this quantity of cytoplasm would disturb the critical cytoplasmic-nuclear ratio and the organism "would be forced to" regenerate the lost cytoplasm prior to division.^{11,14}

In *Blepharisma undulans*, a ciliate species, it was previously found in this laboratory that such daily amputation could not suppress division.⁷ The present studies were undertaken in an attempt to repeat the previous studies on *B. undulans* and also those of *Amoeba proteus* since this species was most successfully used to show that division could be inhibited.^{5,11,14}

The concept involved is an important one in biology since it postulates an inability of an organism or cell to divide unless the precise conditions for division are satisfied. These stipulations would require a doubling or replication of the requisite factors in the nucleus before division could occur. The replication of the nuclear components in turn would be dependent upon correct amounts and kinds of critical cytoplasmic factors.^{9,10}

Support for this critical relationship as evidenced by divisional suppression is found in the work of Hartmann and Phelps.^{2-5,11} A critical evaluation of the early work is found in the review by Adolph.¹

Detailed analyses on *A. proteus* made by D. Prescott¹²⁻¹⁴ further elaborated this nuclear-cytoplasmic relationship. Excision of a critical volume of cytoplasm in interphase animals was correlated with reduction in nuclear volume and suppression of cell division.

Materials and Methods

A. proteus was grown on Brandwein solution and fed *Chilomonas* sp. and *Colpidium* sp. in rice infusion. Mass cultures, grown in agar-lined finger bowls, were used and organisms in divisional and postdivisional stages were selected for the cutting experiments.

Attempts were made to determine nuclear-cytoplasmic volumes with the use of a rotocompression chamber, the compression proved damaging to the organisms and was abandoned.

Cloned *Blepharisma* were grown with *Pseudomonas ovalis* in lettuce and Brandwein solution.

Cutting of both species was continued for five and six days.

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Isolated *B. undulans* and *A. proteus* were used for the cutting experiments. They were cut freehand using glass needles and isolated in agar-lined 12 spot Kline slides, and fed daily.

Groups were set up as follows. For *Blepharisma undulans*: 12 uncut con-

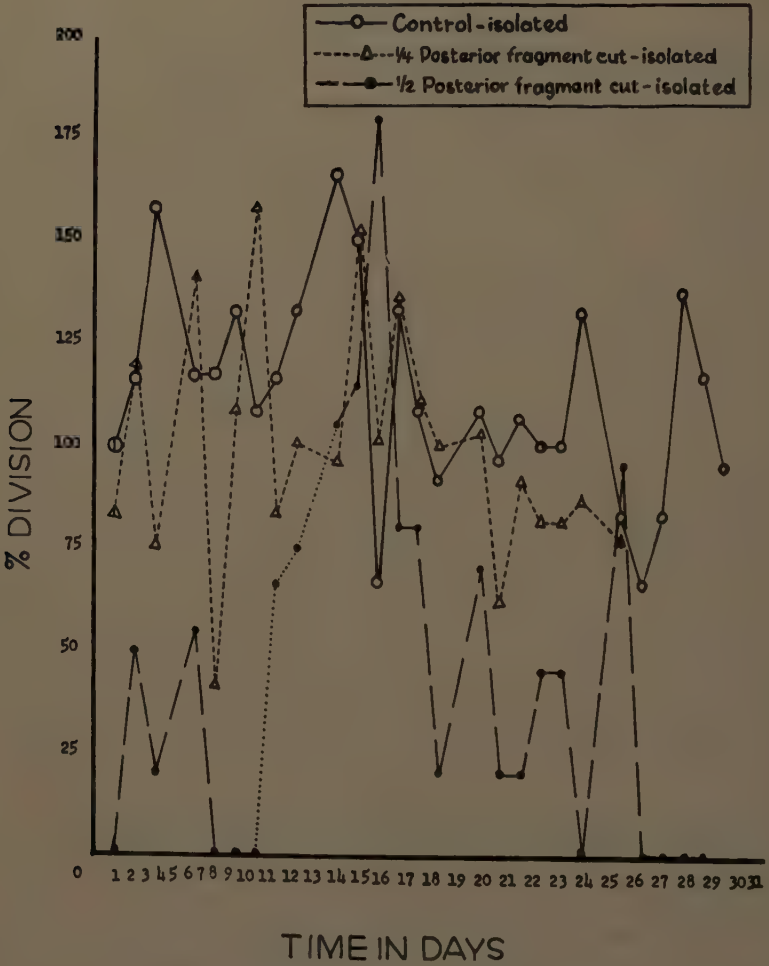


FIGURE 1. Division rate of *Blepharisma undulans*.

trols, 12 with one-fourth of cytoplasm removed daily, and 12 with one-half of cytoplasm removed daily. For *A. proteus*: 12 uncut, untransferred (isolated into Kline slides and then fed daily); 12 uncut, daily transferred into freshly prepared Kline slides; 12 "sham" cut (needle was pressed into the cytoplasm and then kept there until a deep groove could be observed); and 12 with one-fourth to one-third of cytoplasm removed daily.

All groups were maintained at 20° C.; however, difficulties in temperature

regulation were encountered. Three series were done on both species. For details of freehand cutting and making of the needles, see Hirshfield.⁶

Results

The results obtained with *B. undulans* in these studies are comparable to those obtained by Hirshfield and Pecora.⁷ Since the latter's data were taken over a much longer period the graphs (FIGURES 1 and 2) are from that work.⁷

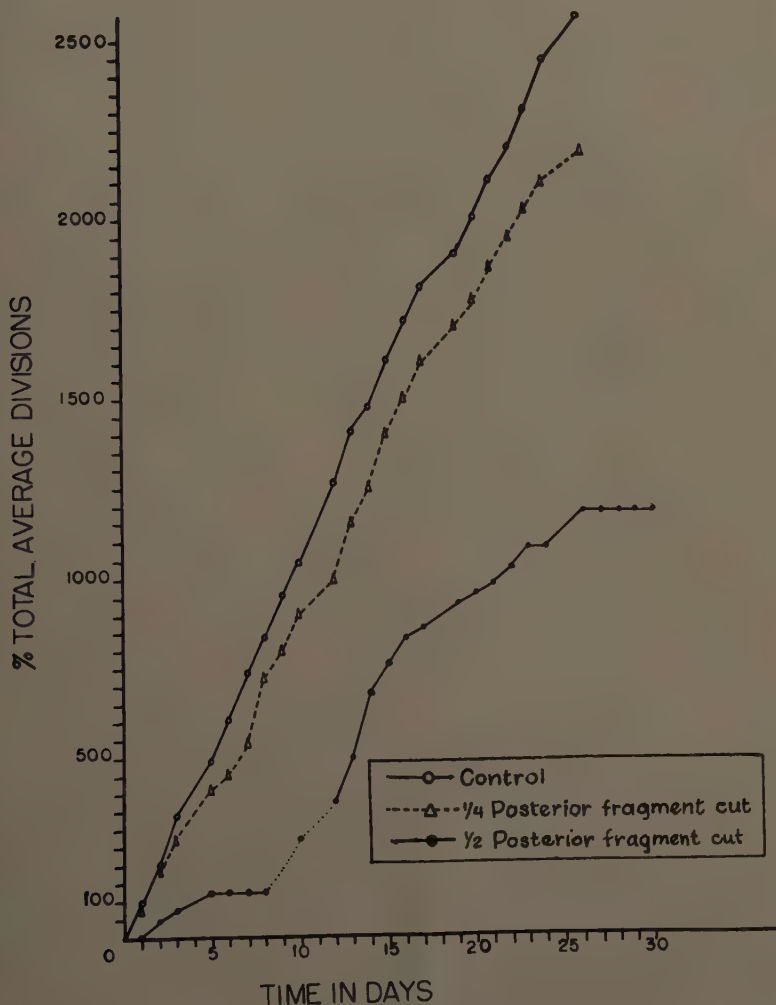
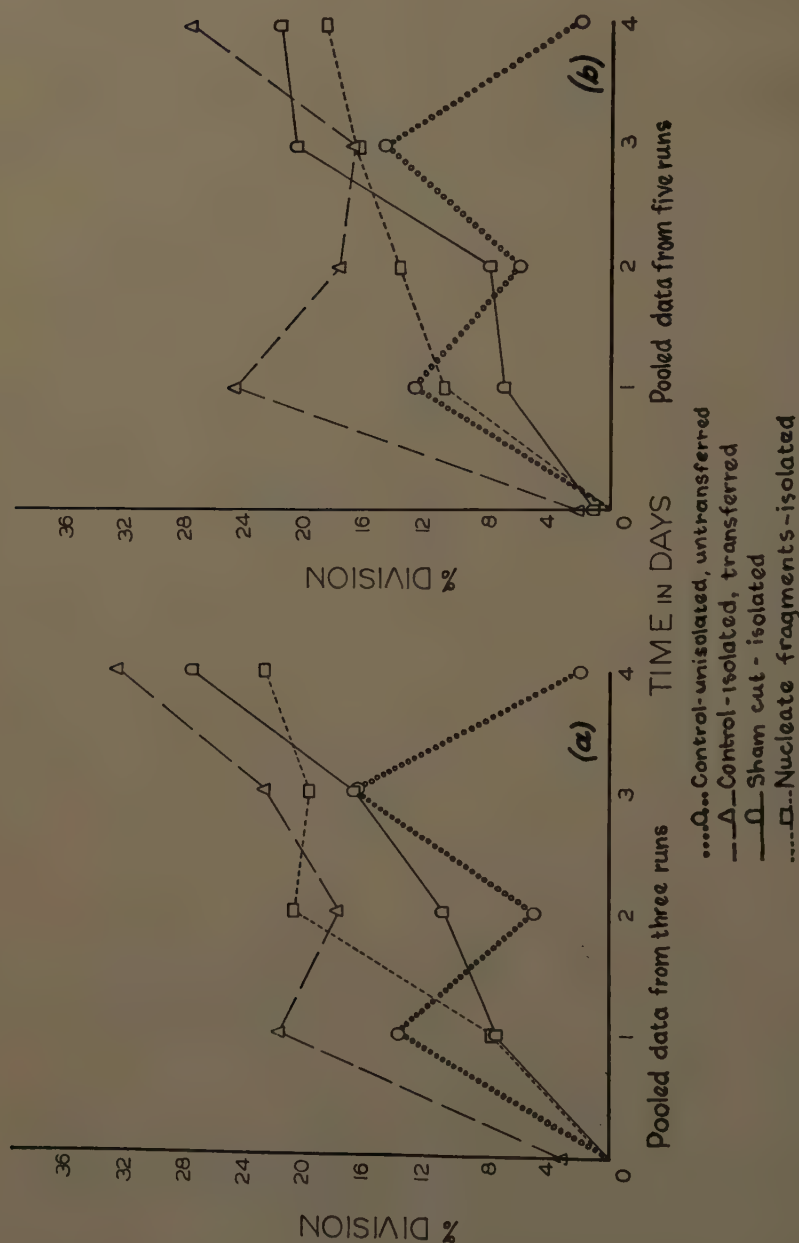


FIGURE 2. Division rate of *Blepharisma undulans*.

The results for this strain of *A. proteus* are given in FIGURES 3a and b and FIGURE 4. No essential difference was found in the experimentals and controls under the above conditions except for a possible lag as compared to the uncut daily transferred forms.

FIGURE 3. Division rate of *Amoeba proteus*.

Discussion

The results obtained in this study are by no means conclusive. Difficulties were encountered in temperature regulation, toxic dish effects, and low division rate of the amoebae (one in approximately 48 hours for *Amoeba*, and one in 24 hours for *Blepharisma*). In previous studies one division per day for *Amoeba* and two divisions per day for *Blepharisma* were obtained.⁶⁻⁸ In addition, cultural conditions, for example, the food organisms for the amoebae, may not have been adequate for optimal growth. Prescott¹⁶ obtained his results on Tetrahymena-fed strain of *A. proteus*, whereas ours could not be maintained on Tetrahymena.

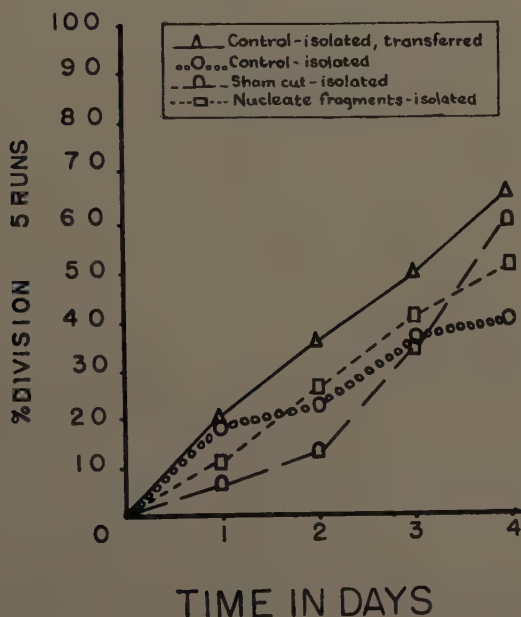


FIGURE 4. Summation curve first division *Amoeba proteus*.

Prescott in 1959¹⁵ pointed out that division in *Amoeba* was probably not purely dependent upon a critical nuclear-cytoplasmic ratio.

The division of *B. undulans* in the one-half cut forms indicates that size, per se, is not crucial. Furthermore, that these forms could be cut daily only for a relatively few days without lysis of the organisms, points to the importance of other factors than nuclear-cytoplasmic ratio since, in these one-half cut forms, the ratio of the amount of macronucleus to the amount of cytoplasm was roughly the same as in the controls.

There is a further possibility that in the one-fourth cut forms in both species where a noticeable lag in division occurs, a nuclear-cytoplasmic interrelationship may be present, whereas in the one-half cut *Blepharisma* other factors imposed by daily transection may be involved. It is rather premature at this time to invoke the role of RNA, DNA, or other cellular components in these

types of experiments without specific information about the biochemical events that are involved in nuclear-cytoplasmic relationships.

In the case of *A. proteus* the similarity of the division rate of the sham cut forms to the cut forms indicates that *A. proteus* may be affected by drastic manipulation.

It is difficult to evaluate the effects of these environmental factors on the results obtained in these studies. However, further studies are in progress utilizing such factors as nuclear-cytoplasmic ratios and amputations of differing quantities of cytoplasm.

The results obtained in these studies indicate that division in these two protozoa is probably based on more complex factors than nuclear-cytoplasmic ratios.

Summary

(1) Daily amputation of *B. undulans* and *A. proteus* did not suppress the division of the organism and does not support the concept of a critical nuclear-cytoplasmic volume ratio.

(2) A lag in division of the daily cut forms as compared to the controls was observed.

(3) "Sham-cut" *Amoeba* gave essentially the same results as cut *Amoeba*.

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ON THE PHYSICAL PROPERTIES OF THE MITOTIC SPINDLE*

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Observation on the birefringence of the mitotic spindle in living dividing cells^{5,8,10,12} combined with Östergren's observation on *Luzula*,¹¹ with Chamber's⁴ and Carlson's³ microdissection experiences and with the anisodiametric "Brownian motion" observed by Bělár² and Bajer,¹ has led to the notion that the spindle possesses a longitudinal fibrous organization with a labile "thixotropic property" (for an excellent general review, see Schrader¹³). Further observation with polarized light of living spindle under various environmental conditions⁶⁻⁹ (also unpublished data) leads to the following physicochemical model of the structure and action of the spindle.

The kinetochore, and the centrioles when present, are centers of crystallization of the spindle and aster micelles. Whereas the centrioles are active in all directions, the activity of the kinetochores is ordinarily polarized. The spindle micelles crystallize into thin filaments from any pole or between two exposed poles and form astral rays and spindle fibers. The aligned micelles are bathed in a pool of randomly oriented micelles that are slightly more hydrated. The less hydrated oriented micelles are in an equilibrium with the nonoriented material and the equilibrium constant is temperature sensitive.⁹ The oriented micelles may be slightly crosslinked but the degree of orientation and crosslinking is a sensitive function of pH and ionic strength. As a consequence, fixation and isolation readily shift the orientation equilibrium and/or crosslinking. The various mitotic movements of chromosomes are then explicable in terms of a change of the delicately balanced equilibria that also involves shifts of water balance.

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NUCLEAR-CYTOPLASMIC RELATIONS IN THE MITOSIS OF SEA URCHIN EGGS*

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Hartmann (1924, 1928), Phelps (1926), and Prescott (1956) have reported an inhibition of mitosis in amoeba by repeated cytoplasmic amputation. These observations suggest either a direct cytoplasmic control of mitosis or an interaction between the cytoplasm and the nucleus leading to an inhibition of the nuclear cycle. However, this pattern of division inhibition does not appear to be applicable to the ciliate *Blepharisma undulans* (Hirshfield and Pecora, 1956).

In order to test further the generality of the hypothesis that the removal of a significant amount of cytoplasm leads to mitotic delay, we have examined the first division cycle of the sea urchin egg.

A small regulative egg appears to be the logical extreme for a test organism, because there is no net cytoplasmic growth and a normal interphase may be lacking. The relatively constant lengths of the second, third, and fourth mitotic cycles (for example, Harvey, 1956) indicate that the amount of cytoplasm per se does not determine the division rate.

Materials and Methods

Gametes were obtained from the sea urchin *Arbacia punctulata* by 12 v A.C. stimulation. The eggs were washed three times in filtered sea water.

Unfertilized eggs were cut into fragments of varying sizes with fine glass needles. Nucleate fragments and control eggs were transferred with a braking pipette into a single field of a compound microscope and inseminated with a minimum dilution of sperm. A three cm. thick CuSO_4 solution was used as a heat filter for the microscope lamp. Camera lucida drawings were used for the measurement of the diameters of the eggs, and the division time of each egg was recorded on the drawing.

Fertilized eggs could be cut without injury when the elevation of the fertilization membrane had been prevented by nine minutes prefertilization treatment with 0.01 per cent crude protease. In some experiments it was necessary to draw the fertilized demembrated eggs into fine braking pipettes in order to elongate the cells for easier cutting. As a control some cells were elongated but not cut.

Results

Reduction of the cytoplasmic volume of unfertilized eggs. Seven separate experiments on the effect of removing cytoplasm prior to fertilization revealed no change in the division time. Some of the experimentally prepared cells were only one thirtieth of the volume of the control cells. FIGURE 1 shows the re-

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sults of an experiment in which the time of division of each member of the total population is compared with the largest cells and those that contained less than 25 per cent of the control volume. No differences can be detected between the whole population and the groups of largest and smallest cells.

Removal of half of the cytoplasm during the first mitotic cycle. In 8 experiments fertilized eggs were cut into approximately equal halves at different times during

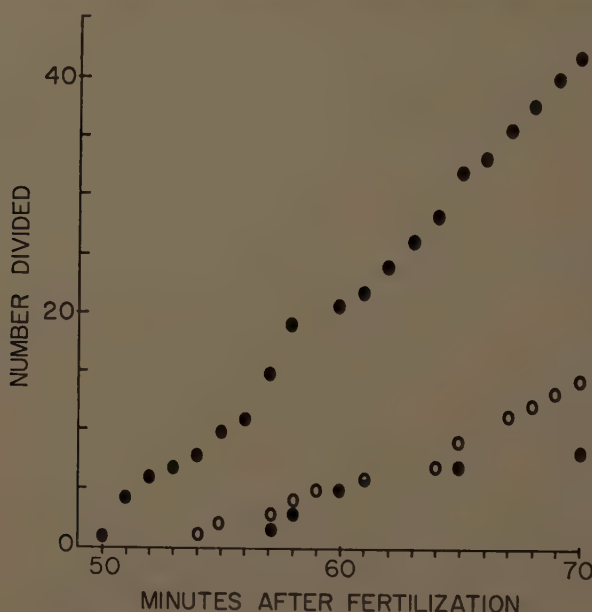


FIGURE 1. Division times of cut sea urchin eggs. The whole population (upper solid dots) is compared with cells containing 90 per cent or more of the control volume (circles) and with cells with less than 25 per cent of the normal volume (lower solid dots).

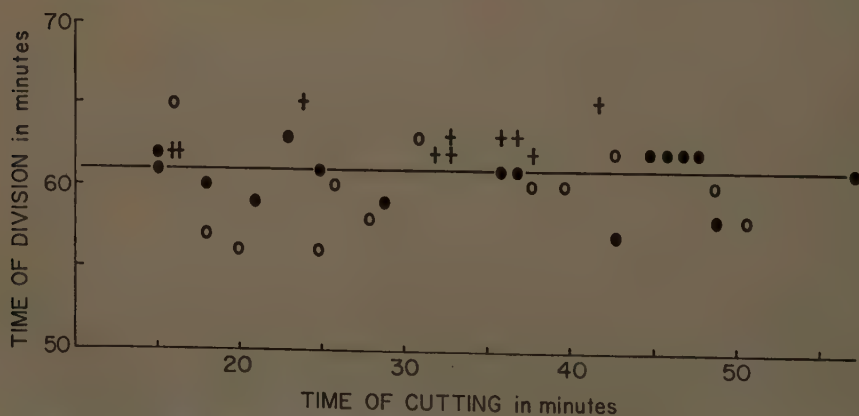


FIGURE 2. Division times of cells cut in half at various times during the first mitotic cycle. Pooled data from three experiments with a 60-min. division average.

the first division cycle without changing the division time. No cells were cut until 15 min. after fertilization in order to allow the pronuclei to fuse.

Since the number of cells that can be cut during a cycle is small, the results of 3 experiments have been pooled in FIGURE 2. The average division time of the proteased control cells was 60 min. The data show that the time of cutting had no effect on the division time. A visual "best fit" line suggests that the average division time was 61 min., which is not significantly different from the control cells. No portion of the mitotic cycle was selectively sensitive.

Discussion

Cytoplasmic amputation either before fertilization or at various times during the first mitotic cycle of the sea urchin egg does not delay mitosis. Hence, removal of part of or even most of the cytoplasm does not lead to any reorganizational activities that delay division. These experiments are consistent with the observation that the second, third, and fourth mitotic cycles have a similar length (Harvey, 1956).

These simple data not only provide an extreme example of the independence of division time from cytoplasmic volume change, but also may introduce a series of qualifications concerning other division properties.

If cells as little as one thirtieth of the normal volume can divide on schedule, then the unit consisting of a fusion nucleus and a small amount of cytoplasm is a well-regulated system. Both the stored materials and the synthetic machinery necessary to initiate first division must be present.

Questions arise as to whether or not the mass of certain cellular systems would vary with the cytoplasmic volume. For instance, the surface area varies as the square of the cell diameter, while the volume varies as the cube of the diameter. The mitotic apparatus is not a geometrically simple structure; hence, its proportions may be different in small cells. The equality of division times suggests that the nucleus-associated structures require little energy or that the energy sources are concentrated near the nucleus.

Reorganizational activities. For simplicity we may classify any alteration in the normal progression of biosynthetic activities in the mitotic cycle as a reorganizational activity. If such activities occur in the sea urchin egg after cytoplasmic amputation, they do not change the length of the mitotic cycle. Similarly, in *Blepharisma* there is no postamputational mitotic rate change (Hirshfield and Pecora, 1956).

In *Stentor* (Hartmann, 1922) and *Amoeba* (Hartmann, 1924, 1928; Phelps, 1926; Prescott, 1956; Hirshfield *et al.* elsewhere in these pages) mitotic delays occur in amputation experiments. In *Stentor* part of the chain of nuclear beads was amputated along with cytoplasm and macronuclear regeneration was found to be necessary for division. A significant feature of the data of Hirshfield *et al.* (elsewhere in this monograph) is that sham-operated amoebae showed a retardation of mitotic rate equivalent to the delay of the cells that had lost cytoplasm. Hence the analysis of nuclear-cytoplasmic relations in amoeba requires further examination with the cutting and handling variables rigorously controlled.

According to Adolph (1931) several earlier workers reported a decrease in nuclear volume in protozoans after cytoplasmic amputation, while others found none. Prescott (1956), who pointed out the errors in measuring the irregular amoeba nucleus, found a consistent trend for nuclear volume loss in two amoebae that were cut and measured daily. If the cell were set back in its mitotic cycle, a compensatory nuclear volume loss is an attractive idea, because in at least four cell types most of the nuclear enlargement occurs late in the cycle (c.f. Adolph, 1931; Prescott, 1956). However, nuclear enlargement occurs in the sea urchin egg, but removal of part of the cytoplasm does not delay mitosis.

An increase in nuclear volume during mitotic delay is a typical result of a variety of experimental treatments (c.f. Brachet, 1957). James (1959) found a small but reproduceable nuclear dry mass loss in amoebae after a temperature shock. While the immediate effects have not been examined, a marked increase in nuclear volume occurs in the sea urchin egg during the course of mitotic delay resulting from a single temperature shock (R. C. Rustad, unpublished). The loss and abnormal gain of nuclear material will require more investigation before any generalizations may be drawn.

Data reported (Phelps, 1926; Prescott, 1956) from three amoebae indicate that a cell that has had part of the cytoplasm removed grows more slowly than a control cell. By means of precise Cartesian diver measurements, Prescott (1955) has shown that the reduced weight of amoebae increased rapidly just after division and then the rate of increase slowed progressively during the mitotic cycle. If a cut amoeba were set back to an earlier time in the normal cytoplasmic growth cycle, it would be expected to begin growing again at a greater rate. The predicted result of a series of cuttings would be the addition of more cytoplasm in the experimental cells than in the controls. Since the growth rate is restrained after cytoplasmic amputation, the amoebae are not being set back to an earlier growth stage. Some reorganizational activity must result from the cutting treatment.

Existing data do not lead to any clear hypothesis of the regulation of cell division by interactions between the nucleus and the cytoplasm. The results of studies on the effects of cytoplasmic amputation are open to several interpretations and biochemical data are not available. Perhaps, as a working hypothesis, it would be reasonable to propose that the cutting treatment can lead to reorganizational activities in cells that have a nonlinear cytoplasmic growth rate.

Summary

Neither prefertilization volume reduction nor amputation of half of the cytoplasm during the first mitotic cycle of the sea urchin egg changes the time of cell division. The problems of nuclear-cytoplasmic relations in cell division are discussed with a comparison of this study to data on growing cells.

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THE ENERGY REQUIREMENTS FOR MITOSIS*

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Introduction

Metabolic studies on the availability of energy for cell division, first made on dividing sea urchin eggs, have shown that aerobic conditions along with efficient respiration and carbohydrate utilization are necessary for mitosis (Krahl, 1950; Swann, 1957). On the basis of these studies the theory originated that the energy for division must be built up before the cell enters mitotic prophase (Bullough, 1952; Brachet, 1957; Swann, 1957).

This theory was extended and elaborated by W. S. Bullough in his reports on mouse ear epidermis cultured *in vitro* (Bullough, 1952; 1955a). Bullough has tried to show that the controlling mechanism for mitosis lies in the accumulation of energy from glucose during a "critical" period prior to visible prophase that he has called antephase.

The present report represents a further investigation of the energy relations for mitosis in mouse ear epidermis cultured *in vitro*. It is our purpose to re-examine the entire concept of antephase in an attempt to arrive at some confirmation or greater elucidation of the present theories on the energy requirements for cell division.

Methods

Mitosis in mouse ear epidermis *in vitro* was studied according to the methods devised by Bullough and Johnson (1951a) which are also described in detail elsewhere (Bullough, 1954; Gelfant, 1959a). Adult male mice are used in groups of five. Small pieces of ear are incubated in Warburg flasks in a Krebs-Ringer phosphate buffered saline medium in the desired gas phase at 38° C. Substrates, inhibitors, or other compounds are added to the culture medium in final concentrations as referred to in the text and shown in the tables.

The ear fragments are incubated for 1 hour to allow all mitoses originally present in the epidermis to pass beyond metaphase. At this point, the experimental period begins. Colchicine is tipped into the main vessel of the Warburg flask and incubation is continued further for 4 hours. The effects of various substrates, compounds, or culture conditions on the development of epidermal mitosis are determined in histological sections by counting the number of metaphase figures arrested by colchicine during the 4-hour experimental period.

Observations

A REVIEW OF BULLOUGH'S OBSERVATIONS, THEORIES, AND CONCEPT OF ANTEPHASE

FIGURE 1 summarizes and correlates the evidence that has led to Bullough's general theory of mitosis control (Bullough, 1952; 1955a) in which glucose and its subsequent conversion to energy plays the critical role in controlling mitosis.

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The first set of data shown in FIGURE 1 establishes the basic importance of glucose and of oxygen for mitosis. Epidermal cells actively enter division only in the presence of glucose, and mitotic activity increases in direct proportion to the oxygen tension of the culture medium. The relationship between oxygen tension and mitotic activity and the fact that mitosis is inhibited in the absence of oxygen (O_2) are used as the first line of evidence that glucose is metabolized aerobically to energy necessary for mitosis. The second line of evidence concerns the glycolytic disposition of glucose, and is indicated by the ability of fructose, pyruvate, or lactate to substitute for glucose in support of epidermal mitosis. Indeed, these glucolytic substitutes are superior to glucose in this

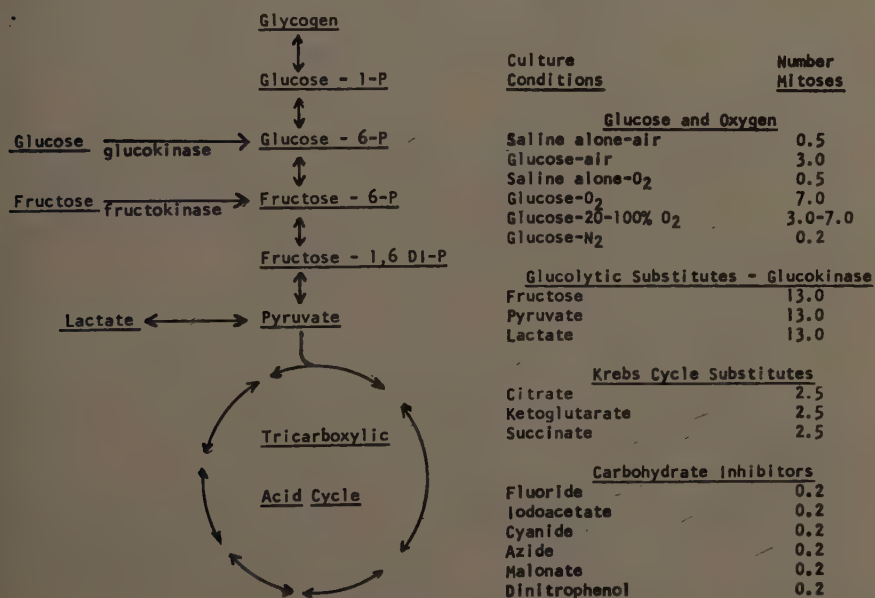


FIGURE 1. A summary and correlation of Bullough's results using mouse ear epidermis cultured *in vitro*. The data listed under "Number mitoses" have been modified and adapted from Bullough's publications (1951a, b; 1952; 1954; 1955a and b) in order to express certain generalizations. See text for explanation.

respect, and information regarding the oxidative metabolism of glucose rests upon the ability of various Krebs cycle intermediates to substitute for glucose to some extent. The last line of evidence in support of Bullough's contention that glucose is converted to energy for mitosis is shown by the effects of carbohydrate metabolism inhibitors. Inhibitors of glycolysis (fluoride, iodoacetate), the tricarboxylic acid cycle (malonate), the cytochrome system (cyanide, azide), or the energy transport system (dinitrophenol) all inhibit mitosis in the presence of glucose.

The next step in Bullough's analysis was the establishment of a single rate-limiting reaction that controls both energy production and mitosis (Bullough, 1955a). The evidence for this theory is also outlined in FIGURE 1. It was reasoned that since fructose, pyruvate, and lactate are approximately twice as effective in supporting epidermal mitosis as is glucose, they are bypassing a

normally rate-limiting step in glycolysis, namely the initial phosphorylation of glucose to glucose-6-phosphate. Thus the glucokinase reaction was conceived to be rate-limiting in its control of glucose utilization, energy production, and mitosis.

All of this information concerning the energy relations for mitosis culminated in the formulation of a comprehensive concept known as "antephase" (Bullough and Johnson, 1951*b*; Bullough, 1952). According to Bullough, antephase is a critical physiological period preceding prophase during which the energy neces-

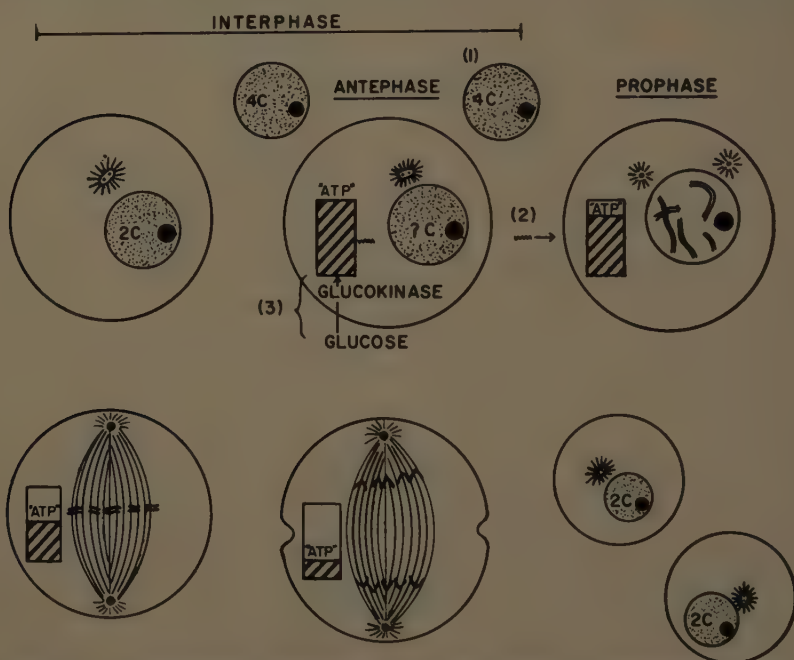


FIGURE 2. A diagrammatic representation of "the concept of antephase" in relation to the classical stages of mitosis. The questions investigated in the present study are labeled in the appropriate position in the upper diagrams by the Nos. 1, 2, and 3. See text for explanation.

sary for mitosis is produced and stored. FIGURE 2 presents a pictorial representation of antephase in relation to the classical stages of mitosis.

Antephase is depicted as a physiological stage during interphase. Extracellular glucose is shown being converted to energy that is presumably stored in the form of ATP, and the glucokinase reaction intercedes in this conversion. The ultimate production of energy from glucose results in a stimulus necessary for mitosis to occur. The cell enters visible prophase, and the original supply of antephase energy is utilized for the activity involved in spindle formation, chromosome movement, and cytoplasmic cleavage.

THE QUESTIONS POSED IN THE PRESENT INVESTIGATION

In view of the widespread acceptance of most of Bullough's manifold theories concerning the energy requirements for mitosis (Ris, 1955; Mazia, 1956; Stern,

1956; Brachet, 1957; Swann, 1957; Wilson, 1959) and the lack of confirmatory studies on mouse ear epidermis, the present investigation undertook to re-examine the concept of antephase and the evidence upon which it was based. The three general problems we studied are labeled numerically and are included in the diagrams shown in FIGURE 2. The first problem concerns the relationship between antephase and DNA synthesis. Since DNA doubling also occurs during interphase (designated by 4C nuclei in the diagram), the question arises whether antephase is related to or includes the period of DNA doubling. The second problem investigated, involves the reputed mitogenic function of antephase energy. Does the energy supply in itself act to initiate mitosis? The third general problem we studied included an examination of the evidence for the conversion of glucose to antephase energy, and for the rate-limiting role of glucokinase in this process.

TABLE 1
ANTEPHASE AND DNA DOUBLING*

Exptl. period (hours)	Saline alone		Saline + glucose	
	DNA doubling (No. 4C nuclei)	Mitotic activity (No. mitoses)	DNA doubling (No. 4C nuclei)	Mitotic activity (No. mitoses)
1	13	0.4	14	2.7
2	12	1.1	18	4.1
3	14	0.7	16	7.3
4	18	1.8	15	8.0

* Adapted from Gelfant, 1958.

Antephase and DNA Doubling

TABLE 1 shows the relationship of antephase energy to both DNA doubling and to mitosis in mouse ear epidermis cultured *in vitro*. Antephase energy has been provided in the usual way by culturing the ear fragments in the presence of glucose and oxygen. DNA doubling was determined by microspectrophotometric absorbance measurements of Feulgen-stained nuclei.

The presence of glucose results in a progressive and marked increase in the number of epidermal cells that enter mitosis during each hour of the 4-hour experimental period. In contrast to mitotic activity, the number of 4C nuclei remained approximately the same both in the presence and absence of glucose. The number of interphase nuclei that leave the 4C condition to enter mitosis is relatively small and would have no significant influence on the number of 4C nuclei measured. Since the over-all proportions of 4C nuclei remained unchanged, it was apparent that antephase energy was not used for DNA doubling. Thus, in answer to the first question, it could be concluded that the antephase period is unrelated to and does not include the period of DNA synthesis.

Antephase Energy as a Mitogenic Factor

The usual method for studying mitosis in mouse ear epidermis *in vitro* makes use of fragments of ear approximately 2.5×5.0 mm. in size. The ears are detached with a pair of scissors and each ear is cut into five small pieces prior

is clearly *negative*. An adequate supply of glucose and oxygen cannot stimulate epidermal mitosis in an ear that remains uncut.

An Examination of the Evidence for the Conversion of Glucose to Energy for Mitosis and for the Rate-Limiting Control of the Glucokinase Reaction

Our examination of the evidence for these contentions follows the original outline and discussion related to FIGURE 1.

Oxygen tension and mitotic activity. The validity of using a relationship between oxygen tension and mitotic activity as evidence for the aerobic metabolism of glucose was investigated by the experiments shown in TABLE 2.

The results in Experiment 1 show that glucose is related to mitotic activity, for in the absence of glucose relatively few epidermal cells enter mitosis: 0.002 *M* glucose represents the optimum concentration for mitotic activity, whereas

TABLE 2
EFFECTS OF GLUCOSE CONCENTRATION AND OXYGEN TENSION ON
EPIDERMAL MITOTIC ACTIVITY *IN VITRO**

Experiment 1		Experiment 2		
Glucose concn. (<i>M</i>)	No. mitoses	Gas phase % O ₂ /N ₂	Glucose concn. (<i>M</i>)	
			0.002	0.02
0.0	0.7	100% O ₂	6.6	5.6
0.0002	1.5	95/5	5.4	3.1
0.001	3.5	80/20	5.0	1.8
0.002	6.2	40/60	6.1	2.8
0.01	3.2	20/80	4.4	1.0
0.02	2.0	100% N ₂	Necrotic	Necrotic
0.05	1.4			
0.2	0.3			

* Adapted from Gelfant, 1959b.

0.02 *M* represents a point of diminishing glucose effect. This observation is pointed out and is of particular significance since the results in the present study are to be compared with those obtained by Bullough (Bullough and Johnson, 1951b) using glucose concentrations of 0.02 *M*. Experiment 2 shows once again that 0.002 *M* glucose is more effective than 0.02 *M*, and that this superiority is maintained irrespective of the oxygen tension in the culture medium. Experiment 2 also shows that a relationship between oxygen tension and mitotic activity depends upon the concentration of glucose in the medium. When the medium contains 0.02 *M* glucose, mitotic activity increases with rising oxygen tension. But when a concentration of 0.002 *M* glucose is used, this correlation does not exist. If a relationship between high oxygen tension and mitotic activity is to be used as positive evidence for the aerobic metabolism of glucose (Bullough, 1952; 1955a), it should, we believe, be shown to exist at the concentration of glucose that is optimum for mitosis. Since this cannot be shown, the sequence of reasoning based upon a relationship seen only in suboptimum conditions is open to question. The final point illustrated

in Experiment 2 concerns the effects of culturing mouse ear epidermis under anaerobic conditions (100 per cent, N_2). To say as Bullough has (Bullough and Johnson, 1951*b*; Bullough, 1952) that mitosis is inhibited under anaerobic conditions (100 per cent N_2) and that this inhibition is related to energy production is to side-step the important fact that epidermal cells become necrotic in this instance (Experiment 2). Our results therefore question the validity of the evidence that has been brought to bear on the relationship between oxygen, glucose metabolism, and mitosis in mouse ear epidermis *in vitro*.

The use of glycolytic and Krebs cycle intermediates. The next area we examined concerned the question of using glycolytic and Krebs cycle intermediates as evidence for the glycolytic and oxidative metabolism of glucose, and as evidence for the rate-limiting control of glucokinase. Our examination of these points is shown by the experiments listed in TABLE 3.

TABLE 3
EFFECTS OF GLUCOLYTIC AND KREBS CYCLE INTERMEDIATES ON
EPIDERMAL MITOTIC ACTIVITY *IN VITRO**

Experiment 1				Experiment 2			
Substrate	100% O ₂		Atmosphere 0.02 M	Substrate	100% O ₂		Atmosphere 0.02 M
	0.002 M	0.02 M			0.002 M	0.02 M	
Glucose	4.2	2.7	1.1	Glucose	2.5	1.6	1.0
Fructose	4.1	4.1	2.8	Citrate	0.3	0.08	0.2
Pyruvate	3.8	3.8	4.4	Ketoglutarate	0.2	0.04	0.04
Lactate	1.9	3.3	5.2	Succinate	0.4	0.2	0.2
				None	0.3		

* Adapted from Gelfant, 1960*a*.

With regard to the glucokinase reaction being rate-limiting in the process of energy production for mitosis, Experiment 1 shows that neither fructose, pyruvate or lactate are in any way superior to optimum glucose (0.002 M) in supporting epidermal mitosis. Thus the crucial observation that these glycolytic substitutes are superior to glucose, and the reasoning that they are therefore bypassing the normally rate-limiting glucokinase reaction do not hold up. Our results help to explain Bullough's observations by showing that in those cases where glycolytic substitutes appear twice as effective as glucose it is only because they are being compared with the effects of suboptimum glucose conditions (0.02 M). Because of these findings the present report questions the significance of the view (Bullough, 1955*a*) that the glucokinase reaction is rate-limiting in its control of energy production and mitosis.

Our study confirms the ability of fructose, pyruvate, or lactate to support epidermal mitotic activity (Experiment 1), but we do not believe as Bullough does (Bullough 1955*a*) that successful substitution by glycolytic intermediates necessarily reflects the glycolytic disposition of glucose in its course to energy for mitosis.

The next point for discussion concerns the use of Krebs cycle intermediates. The results in the present study using citrate, α -ketoglutarate, and succinate

were consistently negative (Experiment 2). In the only other published experiments on mouse ear epidermis *in vitro* (Bullough, 1955*b*), the saline controls were omitted so that the relative effects of the Krebs cycle intermediates cannot properly be evaluated. Thus, in no case has it been shown that Krebs cycle intermediates can support epidermal mitosis *in vitro*. Therefore Bullough's inferences (Bullough, 1955*a*; 1955*b*) regarding the oxidative metabolism

TABLE 4
EFFECTS OF METABOLIC INHIBITORS ON EPIDERMAL MITOTIC ACTIVITY *IN VITRO**

Experiment 1		Experiment 2		
Carbohydrate metabolism inhibitors				
Inhibitor	No. mitoses	Substrate	Alone	IoA or NaF
Glucose control	4.0	None	1.0	0.3
Phloridzin	0.6	Glucose	5.0	0.2
Iodoacetate	0.1	Fructose	4.0	0.2
Fluoride	0.3	Pyruvate	3.0	0.9
Malonate	0.3	Lactate	3.0	0.2
Azide	0.0	Citrate	1.0	0.1
Cyanide	Necrotic	Ketoglutarate	1.0	0.1
Dinitrophenol	Necrotic	Succinate	1.0	0.2
Experiment 3		Experiment 4		
Noncarbohydrate inhibitors		Recuperative capacity		
Compound	No. mitoses	Inhibitor (5 hours)	→ wash	No inhibitor (4 hours)
Glucose control	5.0	Glucose control		2.0
Adenine	0.1	Azide		0.0
Thymine	1.5	Dinitrophenol		Necrotic
8-Azaguanine	0.1	Malonate		0.6
Kinetin	1.5	Iodoacetate		Necrotic
Phenylalanine	0.3	Adenine		0.1
Ethionine	2.0	8-Azaguanine		Necrotic
p-CMB	0.1	Mercaptoethanol		Necrotic
Mercaptoethanol	0.8	100% Nitrogen		Necrotic
DFP	1.5	Carbon monoxide (dark)		Necrotic

* Data from various experiments were combined and modified after Gelfant, 1960*b*.

of glucose, which have been based upon the assumption that Krebs cycle intermediates can support epidermal mitosis, appear highly questionable.

The use of carbohydrate metabolism in inhibitors. The third and last line of evidence we examined involved the use of carbohydrate metabolism inhibitors to prove that glucose is metabolized to a source of energy necessary for mitosis (Bullough, 1952; 1955*a*). Our analysis of this line of evidence is shown by the experiments listed in TABLE 4.

Experiment 1 shows that compounds known to prevent the conversion of glucose to energy do inhibit epidermal mitosis. However, it should be pointed out that these compounds may be toxic, for at relatively higher concentrations they all cause tissue necrosis (illustrated by the effects of cyanide and dinitro-

phenol in Experiment 1). The main question considered here was whether or not mitotic inhibition accurately reflects the metabolic role usually assigned to these inhibitors of carbohydrate metabolism. If the effects of carbohydrate inhibitors were related to energy production for mitosis, one should be able to demonstrate a specificity of action of these compounds in this regard. Our study was therefore directed toward an examination of the specificity of action of these compounds.

The first step in this direction was to see whether the effects of glycolytic inhibitors could be lifted or bypassed by any of the appropriate carbohydrate intermediates. Experiment 2 approaches this problem by testing the effects of iodoacetate and sodium fluoride in the presence of a variety of carbohydrate intermediates. Iodoacetate and sodium fluoride inhibit mitosis in the presence of all substrates tested and severely depress mitotic activity even in the absence of any substrate. Thus the mitotic block imposed by iodoacetate or sodium fluoride cannot be lifted by any of the appropriate carbohydrate intermediates (particularly by pyruvate or lactate). It therefore seems warranted to conclude that the effects of these inhibitors are not due to an inhibition of glycolysis.

Another insight relevant to the question of specificity can be drawn from our experiments using metabolites, antimetabolites, and compounds generally unrelated to carbohydrate metabolism (summarized in Experiment 3). Since most of these compounds, including purine and pyrimidine bases (adenine, thymine), purine analogues (8-azaguanine, kinetin), amino acids (phenylalanine), amino acid analogues (ethionine), sulfhydryl reactants (*p*-chloromercuribenzoate [*p*-CMB], mercaptoethanol), and a cholinesterase inhibitor (diisopropyl fluorophosphate [DFP]) also inhibit epidermal mitosis, it would appear that none of these compounds, including carbohydrate inhibitors, is acting in a specific biochemically defined manner in relation to mitosis in the present system.

The last approach to the question of specificity of action was to see whether epidermis could recuperate from the effects of an inhibitor to the extent that epidermal cells still had the capacity to divide. Ear fragments were cultured for five hours in the presence of a variety of carbohydrate and noncarbohydrate metabolism inhibitors. The fragments were then washed and further incubated for four hours in a medium free of inhibitor but containing glucose and colchicine. The results obtained by this procedure are summarized in Experiment 4. It can be seen that pretreatment with an inhibitor results in an unremitting inhibition of mitosis or in permanent signs of epidermal damage. The inability of epidermis to recuperate after exposure to 100 per cent nitrogen (oxygen lack) or to carbon monoxide (a highly specific inhibitor of cytochrome oxidase) offers additional evidence for the nonspecific effects of carbohydrate inhibitors in the present system. Thus, the effects produced by an inhibitor during the five-hour exposure period, whether on mitosis alone or on epidermal damage, were permanent and irreversible. Such results would seem to indicate that most carbohydrate inhibitors are acting as general toxic agents affecting cell viability rather than energy production for mitosis in mouse ear epidermis *in vitro*.

In view of the foregoing experimental analysis, we conclude that carbohy-

drate metabolism inhibitors cannot be used to prove a relationship between energy production and epidermal mitosis because their effects (in general) are nonspecific, toxic, and irreversible.

Further evidence concerning the role of glucose as an energy metabolite for mitosis. The ultimate observation concerning the role of glucose as an energy metabolite for mitosis rests upon the fact that epidermal mitotic activity increases when glucose is added to the culture medium. If other monosaccharides, some of which are considered to be nonutilizable by mammalian tissues, also increased mitotic activity, the presumed role of glucose as an energy metabolite for epidermal mitosis would then appear questionable. Experiments were therefore designed as shown in TABLE 5 to test the effects of various carbohydrate substrates on epidermal mitotic activity.

In addition to D-glucose, three hexoses and three pentoses permitted the development of epidermal mitosis. Fructose, galactose, and mannose were at least equally as effective as D-glucose in this respect and although arabinose,

TABLE 5
EFFECTS OF VARIOUS HEXOSE AND PENTOSE SUBSTRATES ON
EPIDERMAL MITOTIC ACTIVITY *IN VITRO**

Experiment 1		Experiment 2	
Substrate	No. mitoses	Substrate	No. mitoses
None	0.4	None	0.1
D-glucose	3.2	D-glucose	2.9
L-glucose	0.2	L-arabinose	1.1
D-fructose	2.9	D-ribose	1.4
D-galactose	2.6	D-xylose	1.4
D-mannose	2.8	L-rhamnose	0.6

* Adapted from Gelfant, in press.

ribose, and xylose were not as effective as D-glucose, these pentoses still supported mitotic activity to a significantly greater extent than a medium without substrate. Since mitotic activity is thus also increased by a variety of hexose and pentose carbohydrate substrates, including some (galactose, arabinose, ribose, and xylose) whose ability to be utilized by cells in tissue culture is by no means established (Glock, 1955; Levine, 1957; Eagle *et al.*, 1958; Bailey *et al.*, 1959; Morgan and Morton, 1960), one may indeed question whether glucose actually functions as an energy metabolite for epidermal mitosis *in vitro*.

Summary and Conclusions

Our over-all conclusions with regard to the concept of antephase energy requirements for mitosis are depicted in the diagrams shown in FIGURE 5. The upper series of diagrams shown in FIGURE 5 are taken from our original pictorial representation of antephase (FIGURE 2), and are to be compared with the lower series of diagrams that represent the conclusions developed in the present report.

The first question we asked, whether antephase includes the period of DNA synthesis, was answered negatively by results that show that DNA doubling

is unaffected by conditions that presumably provide antephase energy (glucose and oxygen). DNA doubling takes place before experimental alterations in energy supply during antephase are studied. Thus the DNA content of the nucleus in antephase can be designated as being 4C, as shown in the lower diagram.

The second question of whether the potential antephase energy, supplied in the form of glucose and stored in the form of ATP, acts as a mitogenic factor to initiate mitosis is answered negatively by results that show that cutting of the ear prior to its incubation *in vitro* is the initial and controlling stimulus for

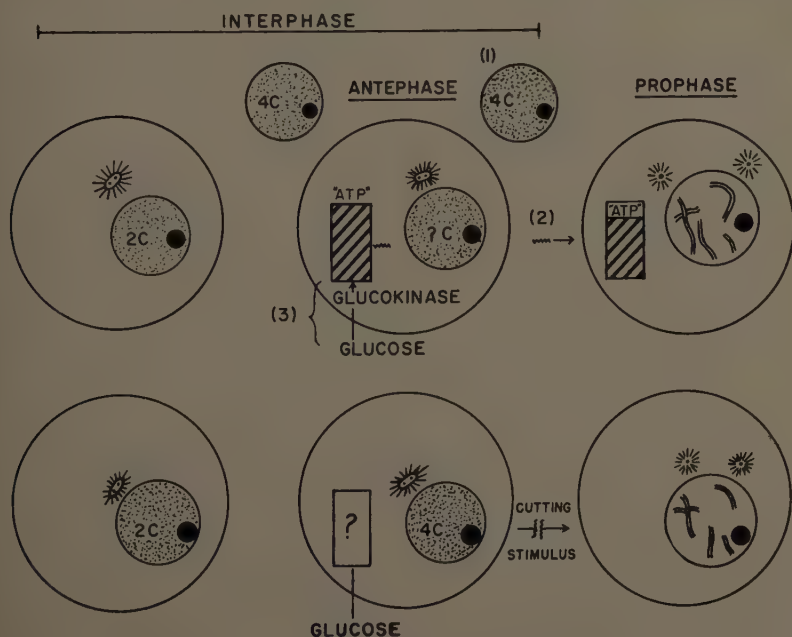


FIGURE 5. The upper series of diagrams of "the concept of antephase" are the same as those in FIGURE 2. The lower series of diagrams represent a summary of the conclusions developed in the present report. See text for explanation.

mitosis. Thus cutting has been established as an independent mitogenic factor, and this stimulus is necessary for mitosis to occur in mouse ear epidermis *in vitro*.

The question of whether the glucokinase reaction is rate-limiting in its control of energy production and mitosis was also answered negatively. Bulbough's evidence in this respect involving the superiority of fructose, pyruvate, and lactate does not hold up when the effects of these glycolytic substitutes are compared with those using optimum glucose concentrations. We have therefore eliminated the glucokinase reaction from the lower diagram in FIGURE 5.

Finally, the question of whether there is any substantial evidence that glucose is indeed utilized as "antephase energy" must be answered negatively. This has been indicated in the lower diagram (FIGURE 5) by eliminating the idea

of potential energy storage (ATP) and by substituting a question mark in its place. Our results show that evidence concerning the conversion of glucose to energy for mitosis cannot be established by a relationship between aerobic conditions and mitosis (there is no relationship between high oxygen tension and mitotic activity in the presence of optimum glucose concentrations; and epidermal cells become necrotic in the absence of oxygen), nor can evidence be established by the use of Krebs cycle intermediates (they cannot substitute for glucose to any extent at all in supporting epidermal mitosis), nor can evidence be established by the prevention of mitosis by carbohydrate metabolism inhibitors (for most of these compounds are toxic, nonspecific, and their effects on mitosis or epidermal damage are irreversible).

Furthermore, our study questions the actual function of glucose as an energy metabolite for epidermal mitosis *in vitro* by showing that nonutilizable sugars can also support epidermal mitotic activity.

In view of these findings it is my opinion that no concrete evidence has ever been presented to show that glucose is actually being converted to, and used as, a source of energy for mitosis in mouse ear epidermis cultured *in vitro*. Since such evidence is necessary to substantiate the theoretical existence of a critical energy-requiring period preceding prophase, the concept of "antephase" as proposed by Bullough (Bullough and Johnson, 1951*b*; Bullough, 1952) becomes subject to question.

The singular and outstanding observation that still remains unexplained is that epidermal mitotic activity increases when glucose is added to the culture medium. The traditional approach to this problem has been to consider glucose as an energy metabolite and to study its function solely in this role. It has already been established that glucose can be metabolized by ear fragments *in vitro*, and even by isolated epidermis (Cruickshank *et al.*, 1955; 1957). However, my concern here is with the mechanism of mitosis and with a theoretical relationship between glucose energy and mitosis in mouse ear epidermis. In regard to this relationship, I have shown the evidence to be inconclusive. For such a relationship to be established, it would have to be shown that glucose provides energy specifically for those epidermal cells that enter mitosis *in vitro*. Since this has never been shown, I submit that the whole problem of the energy relations of mitosis (including the energy-reservoir mechanism theory proposed by Swann, 1957) be reconsidered and opened to further investigation. One new approach might be to set aside the traditional view of glucose as an energy metabolite in order to consider its function in another role: as a compound that has some as yet unknown endogenous effect on the process of cell division.

Indeed, the results currently being obtained in our laboratory indicate that the relationship between glucose and mitosis may be due to an effect on specific cation transport or to an influence on the general osmotic properties of the cell. Therefore it is perhaps by moving into some such new frame of reference that the field of known facts and ideas on the mechanisms of cell division (Swann, 1957; 1958; Mazia (1956) and the others in this monograph) may be expanded.

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CONTROLLED DIVISION SYNCHRONY AND GROWTH IN PROTOZOAN MICROORGANISMS*

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The realization that the occurrence of cell division in mass cultures can be controlled by careful attention to culturing methods has given hope to the possibility of making strong inroads into understanding cell growth and division through the study of biochemical events in the division cycle. It seems, however, as if nature's design for the cell as a self-regulating unit tends to disguise changes in its activity by displaying constancy, the source of which is the statistical behavior of many of its processes. In ordinary culture methods every precaution is taken to insure the constancy of the cellular environment, and cells are obtained whose rate of growth approaches a fixed value in the logarithmic phase. Even the population average of such cells is not constant in its other activities. Average volume, mass, and respiration rate reduced to a per-cell basis may vary throughout this period¹⁻⁴ and, in controlled work, one is always careful to sample cultures at the same population density in the log phase.⁵ The range of nuclear volumes also shows some lack of constancy;⁶ thus the growth rate of a mass culture is a poor measure of the activities of single cells. Despite the constancy of the growth rate in the logarithmic phase, these other factors can definitely be seen to change. The cells in a closed culture in which there is a logarithmic decrease in nutrients and increase in by-products still regulate to a constant average generation time.

Thus the mass culture is well-behaved with respect to this criterion giving a mean generation time that is constant. The constancy of the mean generation time does not, however, imply a lack of variability, and most likely there will be a considerable difference in the dispersion of the distribution of individual generation times for cells sampled and timed from different points in the growth curve. This distribution of individual generation times, which Kendall⁷ has called the *tau distribution*, has been a topic of considerable discussion ever since the classical work of Kelly and Rahn.⁸ The statistics of growth in a mass culture is an intriguing business, and Powell⁹ has tested mathematical models against a large quantity of data obtained on bacterial cells. Powell's attention to the distribution of generation times within a culture has led him to the conclusion that the tau distribution is not a simple normal distribution but follows a Pearson type III model¹⁰ that results from nonlinear behavior of variates. Furthermore, Powell has incorporated this distribution into an idealized age distribution and has compared this model to his findings on cell populations. The agreement is good.

Mathematical statisticians see these problems somewhat differently from cell biologists, and their model of the cell as a device capable of measurable mistakes is refreshing in contrast to an invariant idealized cell. Their view of

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the problem derives from looking at cell behavior as a distribution of events or properties in units of a large population.¹¹ It is a more complete, although more frustrating, point of view. The statistical models are used as touchstones of the real behavior, and the generating functions which produce these models are analyzed for point to point correspondence with the itemized behavior of real populations of cells. For example, the Kelly and Rahn⁸ distribution of generation times in bacteria was thought to be consistent with the accumulation of errors involved in the synthesis of sets of genes, the synthesis of each gene having its own error function. Kendall⁷ proposed an alternate model in which the errors in a temporal series of processes generates the final generation time or tau distribution. Powell¹² has considered both models and concludes that the Kendall model is more consistent with the experimental findings since the gene hypothesis leads to an estimate of gene number that is far too low in the light of other data about gene number.

Modern cell theory recognizes the great complexities of biochemical pathways in cell growth and division and, consequently, a statistical model that would generate the distribution of cellular generation times would need to take into account the probabilities of both temporal and simultaneous reactions in the cell as well as reckon with their interactions. The net usefulness of such a model is thus diluted by the possibility of a choice of too many variables.

Whatever the structural basis may be for the tau distribution, one cannot avoid the conclusion that constancy of the culturing conditions will affect it.¹³ If the generation time could be dissected into age intervals, one should be able to study the distribution of cells in an age interval relative to the average generation time and hopefully discover the source of the variations as a function of cell age. Unfortunately, age markers are not easy to discover. One marker, of course, is the onset of mitosis and its duration, which in turn can be broken into the various mitotic stages including the fission time. Our interest in the tau distribution, the age distribution, and the fission times springs from their application to the topic of this paper.

The Age Distribution and the Fission Time

When attempts are made to synchronize cell division by any one of a number of methods,¹⁴⁻¹⁶ one tries to distort the age distribution of the cells relative to what is normally found in the logarithmic growth phase. The hope is to reduce a population to a model of a single cell. FIGURE 1 shows an age distribution for an idealized system in which the cell generation time is constant and free of variability. In an ideal synchronized population all the cells would, of course, be distributed at one age interval. The combination of areas A and B represents the continuous distribution of cell number plotted against cell age for the range from zero to one generation time, n_i being the number at zero age. An ideal population of cells, all dividing at a constant generation time, would show the number (n_i) of daughter cells at the extreme left, an intermediate number of cells at intermediate ages, and one half the number ($n_i/2$) at the end of the cycle. The negative exponential slope of the curve that subtends the total area is generated by the logarithmic growth of a culture. A simple derivation of this distribution is given in the appendix to this paper.

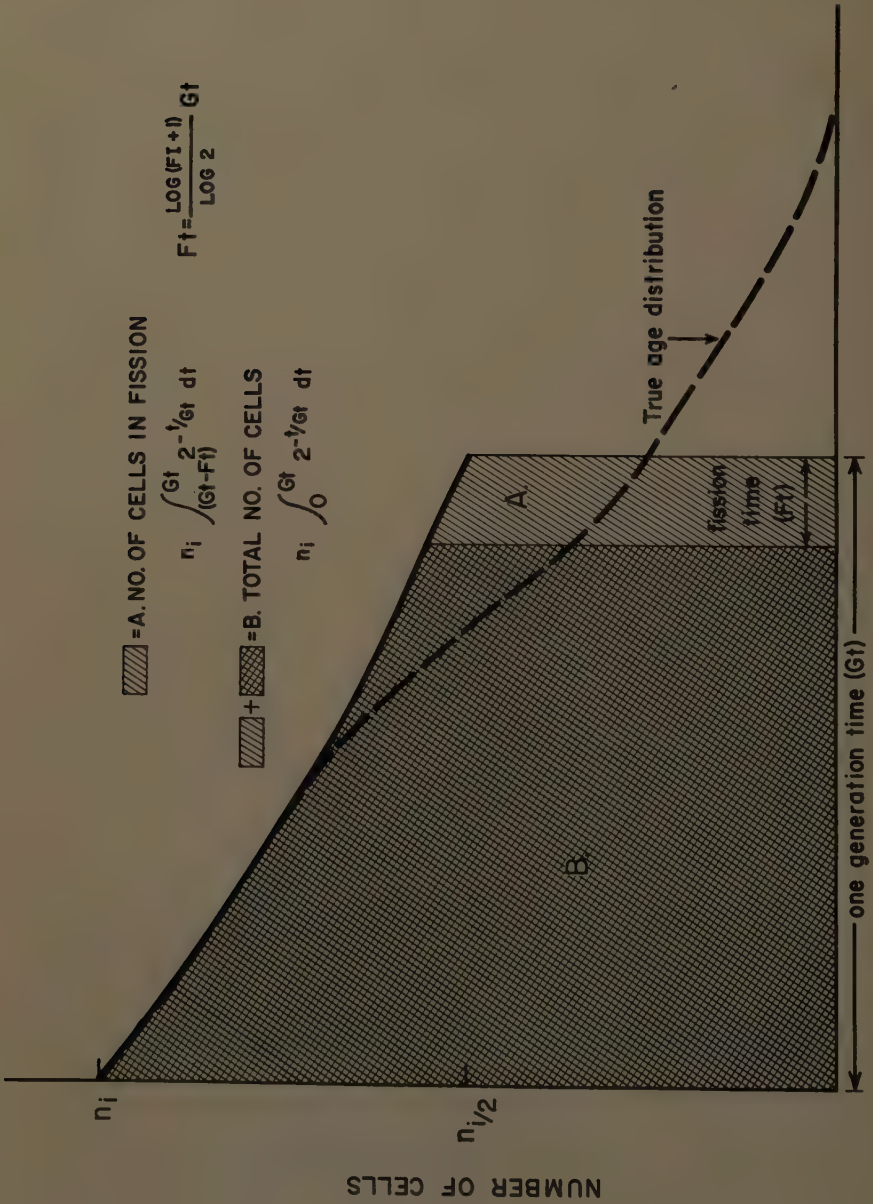


FIGURE 1. Age distribution of an idealized culture showing the relationship between the fission index, generation time, and fission time.

Other derivations can be found in the literature.^{9,17} A representative age distribution found in a real culture is given by the dotted line that continues beyond the point of one generation. The tail, according to Powell,¹² is the result of a variable generation time and would be generated by a tau type distribution plus an ideal age distribution. In practice the idealized age distribution can be used as a first approximation for calculation of the fission time. The area under the total curve, that is, area A plus area B, is equal to the total number of cells. Area A alone is the area that represents the number of cells in fission. On the assumption that the proportion of cells in one age class is constant relative to the total number during growth, the ratio of area A to area A + B gives us the ratio of the number of cells in fission to the total number of cells, that is, the fission index. Solving this relationship and rearranging to obtain the fission time, we see that a formula results stating that the fission time is equal to the generation time multiplied by the ratio of the logarithm of the quantity (fission index + 1) over the logarithm of 2. This equation can then be used to appraise the actual time that the cells spend in fission. This is done on an actively growing culture by determining the generation time and the fission index, and then calculating the fission time. Other formulas for this have been given in the literature,^{18,19} but their derivations have been questioned. A test of this formula has been made in the present instance with the acetate flagellate *Astasia longa* (Jahn), on which we have also done synchronization studies. We have independently measured the fission time of individual cells under the microscope and also calculated the fission time by measuring the fission index and the generation time. We find that our calculated value is very near the observed value. The observed fission time at 20° C. for these cells grown on a balanced salt solution plus acetate, cysteine, and methionine is 11.3 min. $\pm$ 2.6 min. The calculated fission time is 11.9 $\pm$ 1.8 min.; thus the use of the idealized age distribution provides a fair method by which one can appraise the fission time, and the tail resulting from the nonconstant generation time does not destroy this relationship.

Effects on the Fission Index

Our interest in the fission time arose out of the curious behavior of these cells when we attempted to synchronize them on completely defined medium. Our method of synchronizing cells such as *Amoeba proteus*, *A. longa*, and *Euglena gracilis*, var. *bacillaris*, has been to take into account the normal generation time of an organism at various temperatures and to construct a temperature cycle of sufficient length and intensity to permit cells to complete their life cycle in one temperature cycle. In one instance, J. R. Cook in our laboratory has worked with *E. gracilis*, using a light cycle in the same way.²⁰ George Padilla and I first observed that *A. longa*, when grown on a nondefined but sterile medium, proteose peptone* would grow and divide with generation times in the neighborhood of 12 hours at 20° C.⁵ The *A. longa* could be synchronized by a cycle consisting of 15.5 hours at 15° C. and 8.5 hours at 25° C. using one half hour for each transition of the temperature.²¹ The total length of the cycle was 24 hours. A doubling or a near doubling of cells occurred in

* Difco Laboratories, Inc., Detroit, Mich.

the warm period of each cycle, and it could be continued for five or six cycles. However, when a completely defined medium, namely Cramer-Myers medium, was used, synchrony could not be obtained. TABLE 1 gives the composition of this medium, and it may be seen that this organism can grow with vitamins B₁ and B₁₂, the mineral requirements, and acetate. The generation times found on this particular medium were comparable to those found on proteose peptone; yet when we attempted to synchronize cells on this medium, it was next to impossible.

What factor is present in proteose peptone that is not present in the defined medium that will promote synchrony? Our first guesses proved to be rather rewarding. Sulfur compounds, particularly sulfhydryl, were considered, and our first supplements were with cysteine and methionine. By adding these compounds to the basal medium, the cells could be synchronized as readily as on proteose peptone. The early work of Voegtlin and Chalkley²² on the duration of the fission time in *A. proteus* showed that sulfhydryls may play a

TABLE 1
CRAMER-MYERS MEDIUM (MODIFIED)²⁹

Constituents	Concentrations mg./l.	Constituents	Concentrations mg./l.
(NH ₄) ₂ HPO ₄	1000	ZnSO ₄ ·7H ₂ O	0.4
KH ₂ PO ₄	1000	Na ₃ MoO ₄ ·2H ₂ O	0.2
MgSO ₄ ·XH ₂ O	200.	CuSO ₄ ·5H ₂ O	0.02
Na ₃ C ₆ H ₅ O ₇ ·5H ₂ O	800.	Sodium acetate	5000
CaCl ₂	20.	(anhydrous)	
Fe ₂ (SO ₄) ₃ ·XH ₂ O	3.	Vitamin B ₁	0.01
MnCl ₂ ·4H ₂ O	1.8	Vitamin B ₁₂	0.0005
*CoSO ₄ ·7H ₂ O	1.5	pH 6.8	

* Substituted for CoCl₂·6H₂O used at 1.3 mg./l.

role in the reduction of the fission time. The effect of sulfhydryl compounds on the fission time of *A. longa* was therefore investigated.

A Latin square analysis of these effects is shown in TABLE 2. Increasing concentrations of cysteine run horizontally, and increasing concentrations of methionine run vertically. The controls are shown in the upper left-hand corner. The generation time (Gt) in hours, the fission index (FI), and the fission time (Ft) in hours are listed in all squares. The fission time is calculated from the fission index and the generation time. With increasing concentrations of cysteine the fission time gradually decreases from 0.34 to 0.14 hours. For the same range of methionine concentrations, it decreases from 0.34 to 0.18 hours. The concentrations that seem to be most effective are between 10⁻⁴ M and 10⁻³ M. The Latin square data show variability, but the ultimate effect of concentrations in the range of 10⁻³ M is to reduce the fission time to one half or nearly one third of the control. The outlined region of the Latin square is for fission times that have been reduced to a level of 0.25 hours. An effect on the generation time is also evident. These generation times are the shortest we have ever obtained. An average of our other data gives values between 12 to 13 hours.

What is the role of sulfhydryls and related compounds in the reduction of the fission time and why do they play a role in the synchronization of cell division? We have considered three possible modes of action. One may be the effect of these compounds on the oxidation-reduction potential of the medium, a factor important to the growth and division of certain other flagellates.²³ This is based on the idea that some organisms need a partially reducing environment for high growth activity. Second, the organic sulfur compounds may also be involved as chelating agents. Since many heavy

TABLE 2
CYSTEINE

Controls		0 M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M	Average of rows
Gt 10.3 hr. FI 0.027 Ft 0.34 hr.	Gt 10.0 hr. FI 0.023 Ft 0.33 hr.						
Gt 10.6 hr. FI 0.034 Ft 0.49 hr.	Gt 10.5 hr. FI 0.026 Ft 0.38 hr.	11.5 hr. 0.017 0.24 hr.	11.0 hr. 0.026 0.37 hr.	10.5 hr. 0.030 0.45 hr.	9.00 hr. 0.013 0.14 hr.	10.6 hr. 0.024 0.34 hr.	
M Methionine	10 ⁻⁶ M	11.9 hr. 0.033 0.51 hr.	12.0 hr. 0.034 0.56 hr.	10.0 hr. 0.017 0.23 hr.	11.3 hr. 0.028 0.45 hr.	9.70 hr. 0.016 0.21 hr.	10.9 hr. 0.025 0.39 hr.
	10 ⁻⁵ M	10.5 hr. 0.026 0.38 hr.	10.7 hr. 0.028 0.42 hr.	11.0 hr. 0.026 0.40 hr.	10.0 hr. 0.016 0.23 hr.	10.5 hr. 0.017 0.26 hr.	10.5 hr. 0.022 0.33 hr.
	10 ⁻⁴ M	10.0 hr. 0.031 0.43 hr.	10.4 hr. 0.031 0.38 hr.	11.0 hr. 0.026 0.40 hr.	9.40 hr. 0.026 0.25 hr.	9.80 hr. 0.019 0.16 hr.	10.1 hr. 0.022 0.38 hr.
	10 ⁻³ M	8.50 hr. 0.016 0.18 hr.	9.00 hr. 0.016 0.21 hr.	9.50 hr. 0.016 0.22 hr.	9.00 hr. 0.015 0.18 hr.	8.90 hr. 0.016 0.20 hr.	8.9 hr. 0.016 0.19 hr.
Average of columns		10.2 hr. 0.026 0.36 hr.	10.7 hr. 0.024 0.36 hr.	10.5 hr. 0.022 0.32 hr.	10.0 hr. 0.022 0.31 hr.	9.6 hr. 0.015 0.19 hr.	

metals are known to poison the process of cell division, they may reduce the degree of metal poisoning of the mitotic processes. They might thus operate by external action and not enter the cell. The final possibility is that the cell is being supplied with an overabundance of a compound that it normally synthesizes on its own from the culture medium. The normal pathway for the production of these precursors may be rate-limiting to the fission process. The fission time is therefore reduced in the presence of these supplements. This latter point of view would maintain that the sulfhydryl compound becomes involved in some of the biochemical pathways important to the mitotic apparatus, perhaps providing the energy for the polymerizations and depolymerizations necessary to its action.²⁴ A test of a number of compounds to

determine their effects on the reduction of the fission time has been made and is shown in TABLE 3. In this table methionine, cysteine, glutathione, and thioglycolate show an effect, reducing the fission times by one half or greater at concentrations of 10^{-3} *M*. The other two compounds listed, namely serine and ascorbic acid, were used for other reasons. Serine was used as an amino acid control. Hanson²⁶ has established that the amino acids are not used by this organism as an energy source. The serine is structurally related to cysteine in that the OH group is at a position comparable to the SH group on cysteine. The basis for testing ascorbic acid was somewhat different. It was aimed at seeing whether the ascorbic acid-dihydroascorbic acid system might be involved. Ascorbic acid does appear to have an effect at 10^{-3} *M*. We are suggesting that the ascorbic acid pathway important in the sulfhydryl oxidation and reduction similar to that Mapson and Moustafa²⁶ have investigated may be the source of this effect. This preliminary evidence can be no more than suggestive, and we hope to investigate along these lines in more detail.

TABLE 3

Compound 10^{-3} <i>M</i>	Fission time	
	Experiment (Cramer-Myers + Compound) (hours)	Control (Cramer-Myers) (hours)
Methionine	0.18	0.34
Cysteine	0.14	0.34
Glutathione	0.24	0.44
Ascorbic acid	0.19	0.44
Thioglycolate (10^{-6} <i>M</i>)	0.22	0.38
Thioglycolate (10^{-3} <i>M</i>)	0.19	0.38
Serine	0.34	0.28

The thioglycolate, on the other hand, is found to be extremely active. We notice that even in concentrations of 10^{-6} *M* an effect is evident and remains at about the same magnitude at 10^{-3} *M*. Thioglycolate is a compound comparable in structure to acetate, the beta carbon being substituted with an SH group. Since acetate is the carbon source used by this organism, thioglycolate may get into the cell via an acetate pathway. This may account for its greater effectiveness. Thus entry may be necessary for this effect. There is room for considerable experimentation with various sulfhydryls and their inhibitors using this approach. A better approach may be a study of pathways by which these specific compounds are metabolized.

Synchronization Methods

In a consideration of synchronization and the processes that may be involved, it should be emphasized that although a low degree of synchrony was obtained in the early work on *A. proteus*²⁷ the behavior of this organism suggested the importance of matching the temperature cycle to the cell cycle. This is done by successive approximations starting from a knowledge of the generation time as a function of temperature. By selecting one temperature and the time

through which it will act, the other temperature and the time through which it must act become the only variables. Fixing the second temperature fixes the time. These times and temperatures can be approximated from a curve of the generation time versus temperature for the specific organism. Using these values in a cycle will not give synchrony as such, since this would mean that the temporal processes in the cell cycle have the same Q_{10} , a condition known not to exist.¹⁵ We have found in synchronizing both *A. proteus* and *A. longa* that we have had to reduce either one of the times or temperatures to obtain synchrony. This would suggest that the cycle of temperature is more effective in terms of the production of cell number than steady incubation temperature. A similar effect of a light cycle on production of dry weight in algae cultures has been noted by Sorokin and Krauss.²⁸ In the present instance we are reluctant to draw this conclusion since generation times are somewhat variable from one batch of medium to the next. In our original defined medium, which was also used for synchronization, generation time controls gave a 13-hour generation time at 25° C. and a 62-hour generation time at 15° C. Thus 8 hours at 25° C. should have completed $\frac{8}{13}$ of the generation time and 16 hours at 15° C. should have completed $\frac{16}{26}$ of the generation time. The sum of these two fractions, 0.88, is the fraction of the generation time that should have been completed in one cycle, yet the cells divided in one cycle. A similar relationship was found for *A. proteus* with values for this fraction equal to 0.85. Nevertheless caution must be applied in interpreting these findings since, as seen earlier in the Latin square experiments, generation times in the 10-hour range were not uncommon. We recognize the significance of this finding if it eventually proves to be the rule for cells grown on well-constructed cycles. Nevertheless in terms of real efficiency one is not justified in comparing cycled and noncycled cells without first establishing their differences.⁴ In any event, there may be a tendency for the cell cycle to lock in to a temperature cycle.

A. longa grown at 15° C., when moved to 25° C., respire more rapidly on a per cell basis than do cells that are maintained at 25° C. This tends to decrease with time, that is, with subsequent cell divisions. The reciprocal experiment shows that cells grown at 25° C. respire slower at 15° C. than expected, but with time they increase their respiratory rate per cell. In this latter experiment the cells do not divide for approximately 17 hours; thus at the low temperature the number of divisions is reduced to zero and more respiratory machinery is produced per cell. The cells also increased in volume. The cells are thus in a state which on return to 25° C. will be hyperactive with respect to respiration. This excess may then be rapidly channelled into the division processes. This concept of synchronization is based on juggling the capacity and intensity factors of the cell favoring an increase in the respiratory capacity at low temperature and pushing this increase to higher intensity by going to a higher temperature. In a flask culture on a repetitive temperature cycle, seven or eight cycles can be carried out, and there is a stepwise increase in cell number in each cycle. A typical example of this is shown in FIGURE 2. The increases appear to be sigmoid curves. One might ask if there is some relationship between the length of the burst and the tau distribution. Kendall,

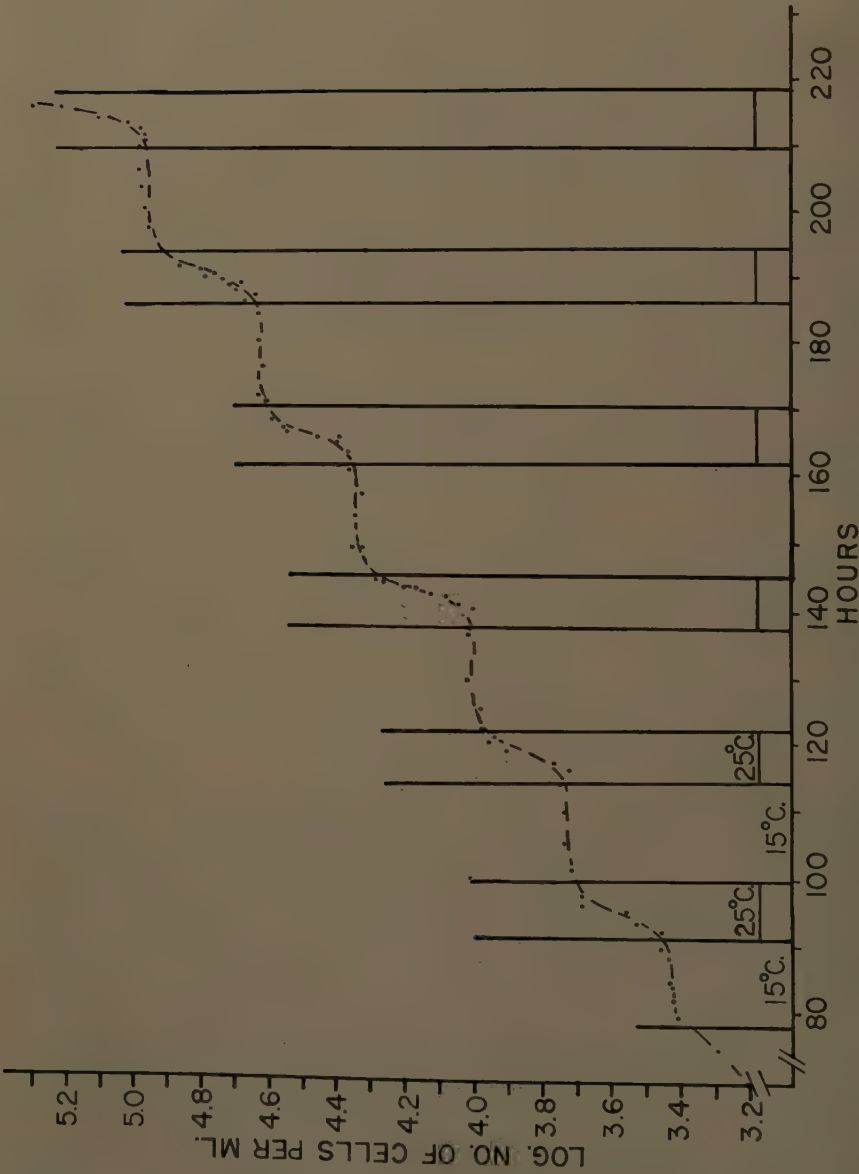


FIGURE 2. Sigmoid division bursts in a synchronized culture of *Asatia longa* on Cramer-Myers medium plus cysteine and methionine.

Powell, and others have considered the tau distribution to be an involved function; however, since we cannot prove that the division burst is a tau distribution no serious error results from considering it a normal distribution. The sigmoid curve of each burst is a cumulative distribution where the function is essentially a sum of successive increases in a given variate with time. If the percentage change in the log number of each burst is plotted on probability

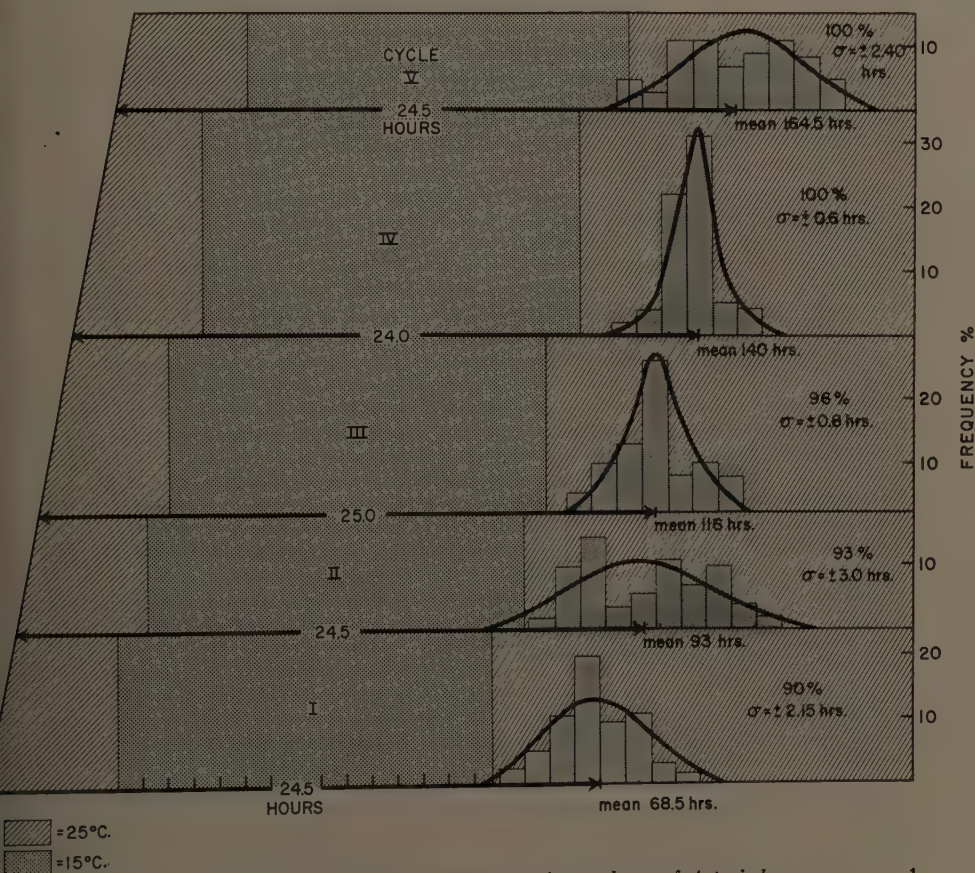


FIGURE 3. Analysis of successive division bursts in a culture of *Aetasis longa* grown on 1 per cent proteose peptone (Difco).

paper, a rather good fit to a straight line is obtained. It should be kept in mind that one is plotting the per cent of the log number and not the per cent of the number. In fact, the per cent number rather than log number does not give a good fit. This point was distressing because it suggested that the burst is disproportional in time with the greatest number of divisions per unit time occurring late in the burst. This would mean that it is distinctly not a tau distribution. The rational basis for this behavior may have its roots in the structure of synchronized populations. We shall discuss it shortly. A series of cycles treated in this way are shown on FIGURE 3. To make this graph

the step curves are plotted each in turn as a cumulative distribution and then from probability paper (probit) fit to a curve and compared to the smoothed curve. The normal distribution and a histogram of the per cent of the log number of cells dividing per hour in time are presented. In this way the effect of cycling a system can be appraised. The mean time of the log number of divisions is shown, and the time between successive bursts is determined between these points in time. Cycles number one to number five show a progressive change in the range of dispersion of each burst and this is shown numerically by the standard deviation for each. It may be seen that the first cycle starts out rather broad and that there is a progressive change in the dispersion of this plot, reaching a highly centralized distribution in the fourth cycle and falling off again in the fifth cycle. Attention should be called to the fact that the areas under these curves are proportional to the per cent of the number of cells that have divided in each burst. These values are subject to the counting error that is kept below 4 per cent by counting a sufficient number of cells. The time between successive bursts is given on the graph and shows how the cell cycle and the mean times between successive bursts obey the 24-hour cycle.

The most interesting part of this consideration is why a log number rather than a number relationship should appear for the burst. Our tentative conclusion is that the synchronization procedure causes a compression of the age distribution. In this instance the term "age distribution" loses meaning because in the course of synchronization cell ages are difficult to discuss. If, however, we assume that the original age classes do not lose their position in time with synchronization but their distance in time from each other is decreased, we can think of their age as identifying them in terms of the order by which they enter the burst. The burst of cell divisions will then most likely display whatever remains of the age distribution, since no new cells will enter the population and the cells will be removed by subsequent divisions in the burst. The number of divisions per unit time in the burst will be derived from the number in each age class as they arrive at the critical point, that is, division. The ideal age distribution shows that there is a logarithmic increase in cell number from left to right in going from the old cells to the young cells. Therefore if these cells divide in that order, that is, oldest cells first and youngest cells last, the number of cells dividing should follow a logarithmic increase. In the real case statistical variation will come into play and the scatter of the log number per unit time should be near normal in its distribution. The mode by which the compression of the age distribution occurs is a subject to which more attention should be paid. For the present we must be content with stating that the degree of compression must be progressively greater for the young cells. This idea would satisfy the generally accepted notion that young cells are less sensitive to treatment than are older cells, especially in terms of blocking their subsequent divisions.

Another consideration of importance is the idea of resonance between the cell and the cycle of temperature. We have emphasized this by stating that when resonance is established one can repeat the burst as many times as the culture conditions will permit. George Padilla, in our laboratory, has carried out such repetition in a quasi-chemostatic system in which he uses a specific

time in the temperature cycle for dilutions of the culture. He has been able to carry this on for two months, obtaining a cell cycle once each day. This is done on supplemented defined medium.

The previous distributions are for a culture grown on proteose peptone. The same calculations made on cells synchronized on the supplemented defined medium are shown in TABLE 4, which gives the mean times at which the bursts occur, the dispersion in terms of a standard deviation in time of the effect

TABLE 4
BURST TIMES AND BURST DURATION OF SYNCHRONIZED *ASTASIA LONGA*

Cycle	Mean of burst time at culture ages (hours)	σ in hours	Time in warm period to the mean (hours)	Time between bursts (hours)
I	71.4	± 3.4	4.4	
II	96.0	± 2.3	5.0	24.6
III	120.0	± 2.5	4.0	24.0
IV	143.0	± 1.2	5.0	23.0
V	166.2	± 1.2	4.2	23.2
VI	190.5	± 1.2	4.5	24.3
VII	215.0	± 1.0	5.0	24.5

Grown on Cramer-Myers medium plus cysteine ($0.58 \times 10^{-3} M$) and methionine ($1 \times 10^{-5} M$).

TABLE 5
LIGHT SYNCHRONIZED *EUGLENA GRACILIS* ON 24-HOUR CYCLE*

Cycle	Cramer-Myers medium		Cysteine and methionine supplemented	
	Mean age at burst $\pm \sigma$ (hours)	Interburst time (hours)	Mean age at burst $\pm \sigma$ (hours)	Interburst time (hours)
I	35.0 ± 2.8		35.0 ± 1.5	
II	60.2 ± 2.6	25.2	59.6 ± 1.5	24.6
III	84.5 ± 3.0	24.3	83.3 ± 1.3	23.7
IV	108.5 ± 2.7	24.0	107.6 ± 1.6	24.3

* Cycle of 16 hours light (130 ft.-candles) and 8 hours dark.

and, finally, the interburst time as measured between successive means. Here again, *A. longa* grown on defined medium supplemented with cysteine and methionine show gradual decrease in the dispersion, although it does not seem to be quite so good as what we have shown for the proteose peptone except toward the end of this cycle. This is one illustration of a tuned system. Further work has been done by J. R. Cook in our laboratory on *E. gracilis* var. *bacillaris*.²⁰ Cook has carried out synchronization studies using a light cycle. This system also shows progressive improvement. In order to determine the best light cycle an estimate was made of the amount of light that was just sufficient to bring cells to division. By doing a growth curve in the light at a given intensity and then moving the cells to the darkness one can establish

how long cells continue to divide. It was found that after 6 hours in darkness the culture leveled off and no further increase in number occurred. The cells that had received just enough light could thus divide some 6 hours later. The generation time of 19.9 hours obtained in constant light could be reduced by 6 hours and still have some cells capable of division. The cycle was designed from this information. Using the approximate 13.9 to 6 ratio, a cycle demanding 16 hours of light and 8 hours of darkness resulted. When this is done division cycling occurs, and a dispersion of the burst time similar to that shown in the previous graphs is seen. The burst time is also decreased by supplementation of the Cramer-Myers medium with the organic sulphur compounds. TABLE 5 shows a comparison of these two situations. The phenomenon seems to suggest that we are dealing with a supplement to the cell's normal capacity for the synthesis of division precursors. Since the effect can be seen on both light-synchronized cells and temperature-synchronized cells, we believe that this is probably a general phenomenon.

Conclusion

The pattern of a synchronous burst is not of the same form as the generalized tau distribution, although the latter may be one of its components. The normal distribution of the per cent log number in the synchronous burst plotted against time leads us to the conclusion that the burst has retained some of the structure of the age distribution. If this is due to a compression of the age distribution, it must be thought of as a disproportionate compression. The young cells under the synchronization treatment must be displaced on the age axis toward the older cells to a greater extent than cells of intermediate age. The action of organic sulfur compounds must further enhance this compression. Their time of action cannot be specified. Detailed attention to mitotic stages may solve this problem. Study of the biochemical pathways by which these compounds are metabolized is definitely warranted.

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APPENDIX

The idealized age distribution present in a logarithmically growing culture can be derived as follows:

Consider first the statement that the total number of cells N_T is equal to the sum of the number in each age class from daughter cells to cells in division, that is,

$$N_T = n_1 + n_2 + n_3 + \cdots + n_n \quad (1)$$

where n is the central value of a class, and n_n is the oldest age class. Consequently, the value of the subscript n will designate the number of these fractions into which the total population is divided. To obtain the proportion of cells in each class, EQUATION 1 is simply divided by N_T

$$\frac{N_T}{N_T} = \frac{n_1}{N_T} + \frac{n_2}{N_T} + \frac{n_3}{N_T} + \cdots + \frac{n_n}{N_T} \quad (2)$$

The next step depends on the assumption that the proportion in each age class will not change with growth of the population. This is equivalent to saying that the age distribution is stable during logarithmic growth of the culture. From this assumption one can see that to know the proportion at any age one must know how the number in a given age class changes as it passes to successive classes as function of time and how the total population varies as a function of time. The best choice of an age class for illustration is the daughter cell class at age equal zero. Since by definition the number in this class will not change for one generation time, we can make use of its constant number remembering that even if the number does not change, the age of this group does as it progressively becomes each age class in succession. The number in this group will be designated as n_i .

Over one generation time in the logarithmic growth phase, the total number increases continuously as expressed by the growth equation.

$$N_n = N_1 2^{n/Gt} \quad (3)$$

N_n is the number at time t_n . N_1 is the total number at the time the daughter cell class is first considered or counted. Gt is the generation time; over one generation, t_n will take on values from $t = 0$ to $t = Gt$. Therefore, the proportion of the population that are daughter cells is $n_i/N_1 2^{t_0/Gt}$ which can be set equal to the first class n_1/N_T in EQUATION 2. Since $t_0 = 0$, this will yield $n_i/N_1 2^0 = n_1/N_T$, and since $N_1 = N_T$, then $n_i = n_1$.

The second term of EQUATION 2 will be equal to $n_i/N_1 2^{t_1/Gt} = n_2/N_T$, and equating term by term in EQUATION 2 we can make the following sum:

$$\frac{N_T}{N_T} = \frac{n_i}{N_T 2^{t_0/Gt}} + \frac{n_i}{N_T 2^{t_1/Gt}} + \frac{n_i}{N_T 2^{t_2/Gt}} + \cdots + \frac{n_i}{N_T 2^{t_n/Gt}} \quad (4)$$

Placing the exponential term above the line and dividing by N_T , the number in each class is determined.

$$N_T = n_i 2^{-t_0/Gt} + n_i 2^{-t_1/Gt} + n_i 2^{-t_2/Gt} + \cdots + n_i 2^{-t_n/Gt} \quad (5)$$

which rewritten as a sum is:

$$N_T = \sum_0^{Gt} n_i 2^{-t_n/Gt}$$

or as a continuous function:

$$N_T = n_i \int_0^{Gt} 2^{-t/Gt} dt$$

Graphically this can be represented as the area under the age distribution of FIGURE 1.

The number of cells in fission will be represented by an area at the extreme right subtended by the fission time (Ft). The fission index (FI), the ratio of cells in fission to the total number, will be equal to the area under this portion divided by the area under the total curve.

$$FI = \frac{n_i \int_{Gt-Ft}^{Gt} 2^{-t/Gt} dt}{n_i \int_0^{Gt} 2^{-t/Gt} dt} = \frac{\left[\frac{2^{-(Gt-Ft)/Gt}}{-\frac{1}{Gt} \log 2} - \frac{2^{-Gt/Gt}}{-\frac{1}{Gt} \log 2} \right]}{\left[\frac{2^{-0/Gt}}{-\frac{1}{Gt} \log 2} - \frac{2^{-Gt/Gt}}{-\frac{1}{Gt} \log 2} \right]}$$

which, when solved for Ft , reduces to:

$$Ft = \frac{\log (FI + 1)}{\log 2} Gt$$

Thus the fission time can be evaluated from the fission index (FI) and the generation time (Gt).

Otto Scherbaum has informed me that an equation similar to the fission time equation appears in a thesis by J. Frankel, Yale University. It was derived by F. Forro.

POSSIBLE SITES OF METABOLIC CONTROL DURING THE INDUCTION OF SYNCHRONOUS CELL DIVISION*

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D. Mazia discusses elsewhere in these pages some aspects of the complex mechanism of cell division as it confronts the contemporary biologist. It is believed at present that at least some of the processes involved in this mechanism can be dissociated and subjected to individual study. This is accomplished by techniques recently developed (for example, synchronization of division), which promise to be of great assistance in re-examining the old question of mitosis or, more candidly, the elusive and controversial "division trigger." For the sake of clarity I shall mention only briefly two prominent concepts concerning this question.

The Growth-Division Interrelationship

The first concept represents the old idea that has often been revived and defended in various forms: namely, that a certain size of a cell is somehow operative as a stimulus for the triggering of cell division. Ideas such as the ratio of cell surface to cell volume and the nucleocytoplasmic ratio belong in this category.

An alternative hypothesis was derived from synchronization studies on the ciliate *Tetrahymena*, in which the processes of growth could be separated from those of cell division. We have concluded that "processes of cell growth and processes leading to cell division may function simultaneously with their own characteristic rates indicating separate but interdependent processes."¹⁸ Admittedly, this idea is not as original as it may sound. It is merely a confirmation of conclusions drawn from observations of variations in size and generation times in *Colpoda*¹ and division delay in synchronously dividing sea urchin eggs.²⁴

The diagrams of FIGURE 1 illustrate these hypotheses, both of which are concerned with the initiation of mitosis. The diagram to the left illustrates what might be called the "switch hypothesis." The length of the life cycle is determined by the growth of the cell between subsequent divisions and the time spent in the fission process.

In the second diagram we also have growth—that is, the necessary duplication of cell material between two subsequent divisions. However, in contrast to the first hypothesis, it is those processes preparing the cell for division, rather than the processes of growth that are primarily responsible for the length of the generation time. I might point out that these preparatory steps for fission occur simultaneously with the over-all growth of the cell.

These two processes, then, are interdependent, as indicated by the arrows in FIGURE 1. However, by recently developed methods we have been able to

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separate and study them individually.²⁰ A felicitous situation is made possible by the inherent nature of what we may call the "mitogenic channel"—that is, the sequence of events described above, which culminate in cell division. The chemical nature of this channel renders it very sensitive to changes in the environment. Consequently, it is not surprising to learn that such diverse means as changes in food or temperature, the application of metabolic inhibitors, or the exposure to radiation (alpha particles, gamma rays, or X rays) can inhibit the cell's preparation for division, leaving growth relatively unaffected. With such diverse means as these at our disposal, we are able to induce simultaneous multiplication in systems, such as algae, bacteria, protozoa,

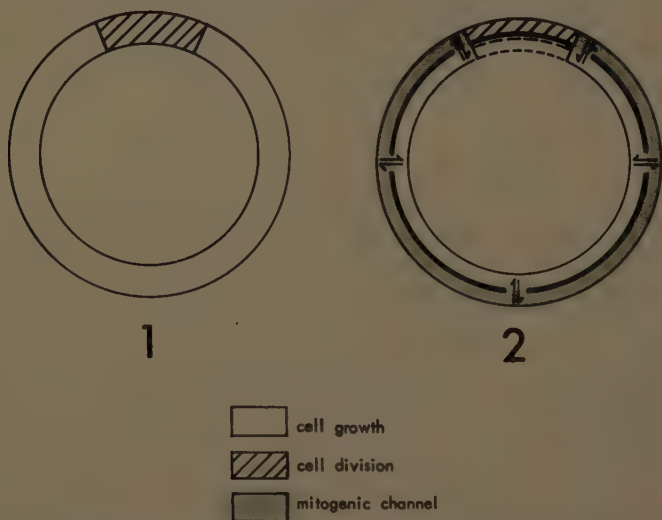


FIGURE 1. Diagrammatic representation of two hypotheses concerning growth-division interrelationships. Further explanation in the text.

and cancer cells. After the successful separation of the growth-division processes, we can proceed to identify their individual nature.

The Experimental Separation of Growth and Division Processes

As an example of such separations I present a few observations on cytological and biochemical aspects of growth in *Tetrahymena*. I shall then make an attempt to identify the chemical nature of the mitogenic channel. These results will be supplemented by work on other synchronized systems as well. At this point the comparative aspects of the phosphate metabolism can be used to greatest advantage. To begin with, I shall give you an illustration of the separation of cellular growth from the preparatory steps for division.¹⁸

FIGURE 2 shows multiplication and growth in a normal mass culture of *Tetrahymena* in a conventional plot of the logarithm of cell number (C_n) and total cell volume (V_n) as a function of time. Upon exposure of such a culture to a series of seven temperature shocks, cell division soon ceases, but cell mass continues to increase for some time. Upon release from the treatment a re-

covery period follows after which almost all the cells divide at the same time (V_h , C_h in FIGURE 2).

Let us now look at three characteristic growth stages in this event. FIGURE 3 represents a sample from a normal exponential multiplication phase. FIGURE 4 shows cells after the heat treatment when no division occurs, but the individual cells have increased in size. In FIGURE 5 we observe the first synchronous division.

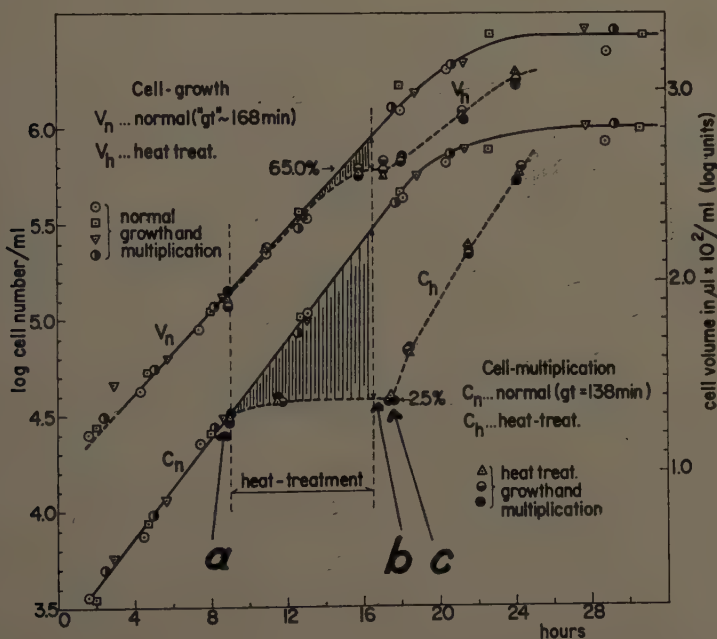


FIGURE 2. Experimental separation of the growth-division interrelationship in a mass culture of *Tetrahymena pyriformis*. Heat treatment consists of a series of seven temperature cycles (each one half hour at 29° C. and one half hour at 34° C.) Further explanation in the text. Arrows *a*, *b*, and *c* indicate the points on the growth curve where the three samples represented in FIGURES 3, 4, and 5 were taken.

A cytological and biochemical analysis of these cells would indicate whether this size increase is due to synthesis of structures and intermediates found in normal cells or constitutes an abnormal response of the cell to the synchrony-inducing agent, as hydration, accumulation of low molecular intermediates or synthesis of macromolecular structures.

Detailed cytological studies revealed that all the cells in an exponentially multiplying mass culture of *Tetrahymena* become arrested in a characteristic stage of their life cycle during this treatment.²⁷ Distinguishable events of duplication of cortical structures in a normal cell are outlined in the following scheme (FIGURE 6). An interesting observation was made in the heat-treated cultures. Counts of the number of kinetosomes along one ciliary meridian and the presence of the anarchic field in all cells indicated a block in the development of these structures at a stage characteristic for a normal cell just



FIGURE 3. Separation of growth-division processes in *Tetrahymena pyriformis* GL, normal culture during exponential multiplication.



FIGURE 4. Separation of growth-division processes in *Tetrahymena pyriformis* GL, normal culture of *Tetrahymena* exposed to seven heat cycles.



FIGURE 5. Separation of growth-division processes in *Tetrahymena pyriformis* GL. the same culture as in FIGURES 3 and 4 during the first synchronous division, approximately 90 min. after the end of the heat treatment. The three samples shown in FIGURES 3, 4, and 5 were removed from the mass culture as indicated by arrows *a*, *b*, and *c* in FIGURE 2.

entering the visible stage of cellular fission. Suffice it to say that biochemical data are available on the composition of the free-amino acid pool and protein-amino acids²¹ and on the base ratios of RNA and DNA in these cells.¹⁹ No abnormal change could be detected in the synthetic pattern of these compounds. For example, the absence of multiplication during the temperature treatment and in the early stationary phase of growth apparently was not caused by a depletion of the free-amino acid pool. One striking observation was excess DNA synthesis prior to synchronous multiplication.²³ Assuming that a strict doubling of DNA occurs between two subsequent divisions, it is puzzling that a *Tetrahymena* cell exposed to the temperature treatment synthesizes more DNA beyond its double amount.

In this connection I shall mention another unusual observation that has no direct bearing on our problem of the biochemical identification of the mitogenic

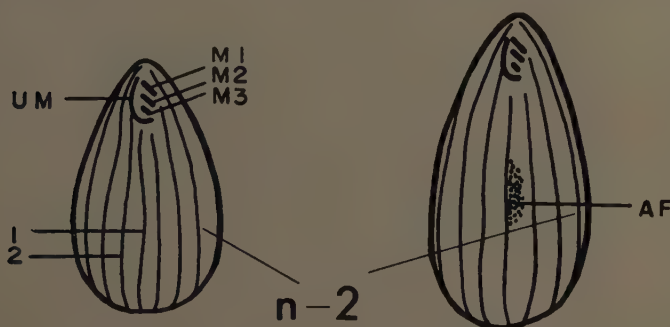


FIGURE 6. Diagrammatic representation of two characteristic growth stages in the normal life cycle of *Tetrahymena pyriformis*. The drawing to the left represents a cell during early interphase, while the one to the right represents a cell in its early cytologically traceable preparation for division. AF indicates the anarchic field, in which kinetosomes accumulate in the formation of the second mouth of the daughter cell. M1, M2, M3, and UM are the four membranellae of the forming mouth: 1 and 2 indicate the first two ciliary meridians. n minus 2 ($n - 2$) represent the meridian along which the kinetosomal counts were made.

channel. The oversized nuclei of heat-treated *Tetrahymena* divide with a shocking inaccuracy and DNA is lost in this process and can be observed as distinct granules in the cytoplasm²² (FIGURE 7). This phenomenon appears also in normal cells and such granules are on the average less than 0.8 per cent of the volume of the newly formed daughter nucleus. In the heat-treated cell this value is 2.8 per cent. The fate of these fragments is unknown. We thought that we might have a unique system and would be able to learn where this material goes simply by labeling the DNA with tritiated thymidine. Unfortunately, it was discovered that the label was also found in the cytoplasm and could not be removed even by acid hydrolysis in the Feulgen treatment or with RNase (O. H. Scherbaum *et al.* to be published). This can be seen in FIGURE 8. However, it could be removed with DNase. Similar observations for other protozoans have been reported.¹³ Since only 35 to 60 per cent of the nuclei were labeled, we felt that growth of these cells with labeled nuclei in unlabeled medium for several generations might shed some light on a possible utilization of this labeled compound in the cytoplasm for later synthesis of

nuclear material. An increase in the percentage of cells with labeled nuclei might indicate that an incorporation of the labeled material from the cytoplasm was occurring. TABLE 1 shows the result of such experiments that do not favor the idea of migration of label from the cytoplasm into the nucleus during growth. These few examples might suffice to indicate that cytologically at least, all the heat-treated *Tetrahymena* cells are blocked at a characteristic stage of their normal life cycle. What can be said, now, about the preparatory processes for division?

Much information on the nucleic acid synthesis in synchronously dividing systems has accumulated in recent years.²⁰ Although the results are not unequivocal, it seems obvious from this work that DNA synthesis, at least, is somehow intimately linked with the subsequent synchronous cell division

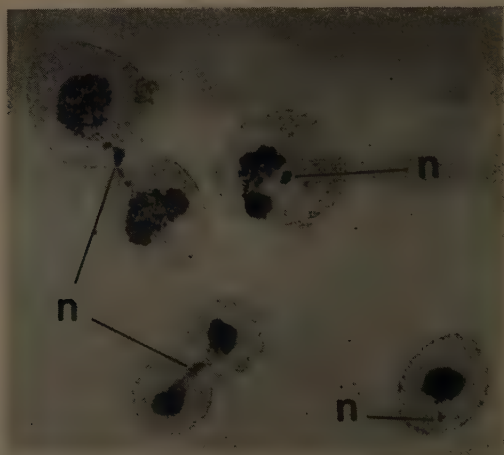


FIGURE 7. Loss of nuclear material during the first synchronous multiplication of nuclear fragments in the cytoplasm during or after cell division (galocyanin preparation).

as indicated in synchronized systems and also observed in normal microbial cultures. For example, on the basis of studies of the effect of azathymine on DNA synthesis and normal cell division of *Streptococcus faecalis*, Prusoff suggests a possible role of a thymine-containing coenzyme in cell division.¹⁶ With respect to synchronously dividing *Tetrahymena*, we found that the DNA content has increased beyond the amount characteristic of a cell prior to fission. That is, an additional synthesis might have taken place during the recovery period between the end of the treatment and the first synchronous division. This is shown in TABLE 2. We do not know, as yet, if this additional quantity of DNA is important for the subsequent division process. A similar increase in the RNA content prior to synchronous cell division has also been observed in *Tetrahymena*.¹⁹ Another connection between nucleic acid metabolism and cell division is found in the yeast *Candida albicans*. If cell division is inhibited by proflavin or tryptaflavin the cells form mycelia with a simultaneous decrease of their RNA content.¹²

Although the role of both nucleic acids in the control of synchronous division



FIGURE 8. Radioautographs of *Tetrahymena pyriformis* labeled with tritiated thymidine. The cells were incubated for seven hours as described in the legend to TABLE 1. Approximately 20 hours after the end of the treatment, when the population had entered the early stationary phase of growth, these cells were removed for autoradiography—the film being exposed for four weeks. (a) The cells were washed in 5 per cent cold perchloric acid. Note that not all cells are labeled in the nucleus: *n* indicates the radioactivity in the nuclear aggregates lost in the cytoplasm during fission. (b) Cells from the same growth stage as in *a*, but subjected to the Feulgen treatment for DNA: *r* marks the only labeled nuclei.

is still enigmatic, it is conceivable that particular RNA and DNA fractions are involved in the synthesis of specific structures required for cell division.

Energy Metabolism

The energy metabolism of growing and dividing cells received much attention ever since Lipmann's pioneering work on ATP. Various phosphate fractions have been isolated from microorganisms and specific roles have been assigned to such compounds in the preparation of a cell for division.

TABLE 1

INCORPORATION OF H^3 -LABELED THYMIDINE DURING THE TEMPERATURE TREATMENT, INDUCING SYNCHRONY OF DIVISION IN *TETRAHYMENA PYRIFORMIS* GL

Stage of population growth	No. of cells counted	No. of cells with labeled nuclei	Cells with labeled nuclei (%)
After seven heat shocks	7620	1914	25.0
One hour after last shock	1232	258	20.9
Two hours after last heat shock	3383	775	22.9
Maximum stationary phase (one day after inoculation)	2949	552	18.7

Cells from the exponential phase of growth (grown in peptone medium) were collected by centrifugation, washed, and resuspended in Elliot's synthetic medium containing 25 μ c./ml. tritiated thymidine. After seven heat shocks the label was removed from the medium by again washing and resuspending the cells in Elliot's medium.

TABLE 2

DNA SYNTHESIS DURING AND AFTER TEMPERATURE TREATMENT IN *TETRAHYMENA PYRIFORMIS* GL

Stage of population growth	Experiment		
	1	2	3
Normal logarithmic phase	17.1 (1.00)	14.8 (1.00)	13.5 (1.00)
After temperature treatment	26.8 (1.57)	26.9 (1.82)	21.5 (1.59)
One hour after treatment, before first synchronous division	30.1 (1.76)	32.9 (2.22)	24.1 (1.78)

Figures give values in μ g. DNA per million cells. Relative amounts in parentheses.

Based on studies of the effect of inhibitors such as carbon monoxide and ether on synchronously dividing sea urchin eggs, Swann proposes a reservoir mechanism.²⁴ Following this hypothesis, energy might be formed continuously during the interphase and then siphoned out at a specific stage in the cell's life cycle when the reservoir threshold has been reached. A similar, more general view is taken by Dean and Hinshelwood with the suggestion that "cell division is triggered off by critical amounts of key substances."²⁴ Hypotheses along these lines of thinking have been used for speculative explanations of cell synchrony in *Tetrahymena*.^{18,28}

It is not as yet clear which compound would fit into such a threshold hypothesis. Plesner reported recently that the average *Tetrahymena* cell during the exponential phase of growth contains 25.3×10^{-6} μ moles of nucleoside

triphosphate.^{14,15,28} This value increases during the heat treatment when no cell division, but growth in mass, occurs. Just 10 min. prior to synchronous division the nucleoside triphosphate concentration was found to be 15 times that in the average cell prior to treatment. Expressed on a relative basis, guanosine triphosphate increases elevenfold while adenosine triphosphate increases only fourfold during the seven hours of exposure to the temperature cycle treatment.

In our own laboratory we observed that the total acid soluble phosphates, including the temperature labile phosphate, of the average log cell increased 1.5 to 2 times during the treatment,²³ but no increase in ATP during this period could be discovered (O. H. Scherbaum and K. Seraydarian, to be published). This is shown in TABLE 3. In all, the determinations (with the luciferin-luciferase system), a significant drop of 33 to 66 per cent was noted in cells exposed to the temperature treatment. Such a decrease is in keeping with

TABLE 3
ATP CONTENT AT VARIOUS GROWTH STAGES

Stage of population growth	Experiment		
	A	B	C
Normal logarithmic phase	5.65 (1.00)	4.12 (1.00)	3.50 (1.00)
Immediately following temperature treatment	2.53 (0.45)	2.79 (0.67)	1.17 (0.34)
One hour after end of treatment, before first synchronous division	1.58 (0.28)	3.70 (0.90)	1.15 (0.33)
Maximum stationary phase (after 24 hours)	1.87 (0.33)	3.96 (0.96)	1.40 (0.40)

Figures give values in μM ATP/mg. dry lyophilized powder. Relative amounts are in parentheses.

the recent discovery of a DPNH oxidase in *Tetrahymena*, which is completely inhibited by mild heating for 10 min. at 40° C.^{5,6} This enzyme appears to be the best candidate as a temperature-sensitive regulator. However, we have no direct evidence as yet concerning the nature of the compound, concentration, or the amount that might be important for the onset of the observable events of mitosis. Unfortunately, the available data on ATP in synchronized *Tetrahymena* cannot be fitted into a reservoir hypothesis. A similar conclusion concerning the role of ATP and phosphates in synchronously dividing sea urchin eggs was reached by Swann from the inhibitor studies that I mentioned earlier.²⁴

At the present time, it seems indicative that insoluble polyphosphates might play a key role in the proposed reservoir concept. Experimental evidence for such a role in synchronously dividing *Corynebacterium diphtheriae* has been provided recently by Sall *et al.*¹⁷ This group exposed mass cultures to cold shocks combined with starvation for 45 min. and upon reincubation in complete medium at the optimal temperature most of the cells divided synchronously through several generations. A several-fold increase in polyphosphate content was observed at the onset of these synchronous fission steps.

Another detailed analysis of the connection between division inhibition and phosphorus metabolism in growing cultures of the yeast *Saccharomyces cerevisiae* is available.⁷ It will be recalled that quite diverse inhibitors, such as alpha particles, gamma irradiation, X ray, nitrogen mustard, and triethylene melamine all produce the same biological effect: inhibition of cell division without an inhibition of growth in mass. The result is formation of paired, enlarged yeast cells. This uniform cytological response was paralleled by a characteristic change in the phosphorus spectrum of these cells: insoluble polyphosphates decreased while the residual labile phosphorus fraction increased. This observation is interpreted by Katchman *et al.* as an enzymatic block in the synthesis of polyphosphates from ATP.⁸ Similar problems of formation and utilization of the polyphosphates in baker's yeast were taken up by Belozerskii and his group, who studied the incorporation of P³² into starved cells.³ Although the mechanism of the utilization of polyphosphates in cells is still unknown, these data are in keeping with other observations that polyphosphates might be used in synthetic reactions preceding cell division.¹⁷

Aside from being a possible source of energy in the "energy pool hypothesis" these polyphosphates might have a control function related to their ability to inactivate enzymes or to form complexes with proteins necessary in the intermediary metabolism.⁷

Possible Sites of Metabolic Control in the Preparation of a Cell for Division

These few examples that I have mentioned on the effect of environmental changes on the nucleic acid and energy metabolism of microbial cells could be supplemented with considerable more data collected elsewhere,²⁰ but factual knowledge is still too meager for an understanding of regulatory changes responsible for the induction of synchronous multiplication.

Although it may seem somewhat premature to venture a working hypothesis this early, we shall not hesitate to do so. One might call this proposal a "reservoir concept" and stipulate that, as yet, unknown compounds must reach a threshold concentration. Let us assume this, for example, with respect to the insoluble polyphosphate fraction (B in FIGURE 9). At a certain concentration of this compound together with other fulfilled requirements such as the presence of soluble RNA and a thymine-containing coenzyme, a new sequence of processes might now be initiated, leading to the synthesis of structures necessary for the known cytological events in mitosis. What evidence do we have for such a scheme and the suggested controlling sites?

(1) The role of nucleic acids in a cell's preparation for division has been mentioned already. In yeast cells, for example, a block at site 8 in FIGURE 9 by the application of proflavin inhibits division, but not growth; and the cells form mycelia.¹² While Wade and Morgan have isolated a soluble RNA fraction from growing *Escherichia coli*, which is lost in stationary cultures,²⁶ Bukhovich and Belozerskii suggest a utilization of ATP or insoluble polyphosphates for the synthesis of RNA in baker's yeast.³ A thymine-requiring mutant of *E. coli* divides synchronously if thymine is added to a culture that has been deprived of this compound for 30 min.,² suggesting a metabolic control at site 3 in FIGURE 9. Prusoff's inhibitor studies with azathymine on

Streptococcus faecalis have already been mentioned;¹⁶ his results also suggest a possible controlling step at site 7 in FIGURE 9.

(2) A site for temperature control is found in the DPNH oxidase system (site 1 in FIGURE 9) of *Tetrahymena*. If we assume a metabolic block at this

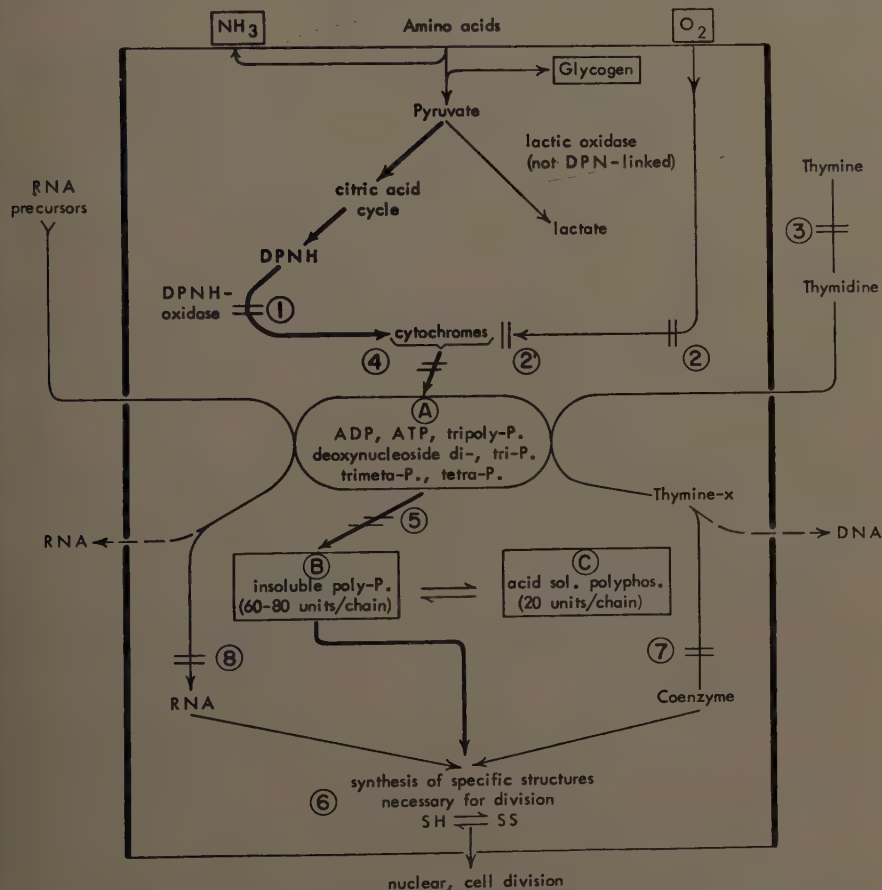


FIGURE 9. Possible controlling sites in a cell's preparation for division. (1) Temperature-sensitive system; see also D. Mazia, elsewhere in this monograph; (2) oxygen-sensitive system, O. H. Scherbaum, to be published; (2') site of inhibition by carbon monoxide and ether (24); (3) specific thymine requirement;² (4) any inhibition at sites 1, 2, 2', or 3 affects size of fraction "A"; (5) radiation-sensitive system, site of chemical inhibition;^{8,27} (6) —SH cycle, site of chemical inhibition;²⁵ (7) formation of a "thymine-containing coenzyme" inhibited by analogues;¹⁶ (8) specific RNA requirement for cell division.^{12,26} (A) residual labile phosphate fraction;⁸ (B) insoluble polyphosphate, possible site of control of cation availability;^{7,8,17} (C) acid-soluble polyphosphates, metabolically inert.⁸ Figures in parentheses refer to the literature; further explanation in the text.

site during temperature treatment a decrease in the concentration of ATP can be expected. This has been observed (O. H. Scherbaum and K. Seraydarian, to be published) although the results are not yet unequivocal. We were able to show that an accumulation of glycogen occurs during the synchrony-

inducing treatment (O. H. Scherbaum and M. Levy, to be published). These observations can be explained by an enzymatic block of DPNH oxidase. The available pyruvate could then be utilized for glyconeogenesis. A considerable accumulation of glycogen has been reported in *Tetrahymena* cultures during the stationary phase when oxygen becomes limiting.¹⁰ In this case, the block might be at site 2 in FIGURE 9 in synchronized cells.

(3) During the recovery period between the end of the temperature treatment and the first synchronous division, adequate aeration is important for successful synchrony in *Tetrahymena pyriformis* (O. H. Scherbaum, to be published). This points to a possible control of synchronous division by the availability of oxygen (site 2 in FIGURE 9).

(4) We suggest site 2' in FIGURE 9 as a site of action of carbon monoxide and ether on the cytochrome oxidase in synchronously dividing sea urchin eggs.²⁴

(5) Due to lack of information on the residual labile phosphate fraction (A in FIGURE 9) and the insoluble polyphosphate fraction (B in FIGURE 9) in *Tetrahymena*, the discussion here is based on work on *Saccharomyces cerevisiae*^{8,9} and synchronized *Corynebacterium diphtheriae*.¹⁷ I have already pointed out that quite diverse agents such as irradiation or certain chemicals control step 5 in FIGURE 9 and in this way control cell division.

(6) The last step in the postulated mitogenic channel consists of the synthesis of specific structures for the division process proper. Whether this process is a synthesis *de novo*, or only a rearrangement of structures already present is an interesting problem. The finding of a significant increase in dipeptidase activity in *S. cerevisiae* at the onset of synchronous budding might be pertinent to this problem.²⁵ Remarkable progress has been made by the isolation of some of these structures from synchronously dividing sea urchin eggs.¹¹ Some aspects of this work are dealt with elsewhere in this monograph (D. Mazia and H. A. Went).

With the help of the proposed working hypothesis in FIGURE 9, we are able to explain certain size changes, length of recovery period after treatment, and some statistical variations in generation times of synchronized *Tetrahymena*, a problem that has been discussed in this monograph by my colleague, T. W. James.

Summary

From the proposed scheme it becomes obvious that adverse environmental changes in nutrition, however mild, including temperature, irradiation, and oxygen tension might not block appreciably the duplication of the cellular structures but may be quite effective in the inhibition of certain synthetic reactions occurring on a "small scale" in a normal cell. These reactions are conceivably the controlling sites in the complex chain of events that take place during the interphase of a cell in its preparation for the spectacular mystery known as mitosis.

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CELL POPULATION KINETICS*

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Cell population kinetics deals with the birth, decay, and other transitions of cells from one state to another. The transition processes are studied in terms of over-all rates involving entire populations; in principle, the single cell is treated more or less as a "black box," an element characterized only by its state and its participation in a transition process. In the laboratory the distinction between the study of individual cells and of cell populations is not a sharp one.

The study of population kinetics has been largely restricted to systems comparatively easy to investigate, that is, those in a steady state. They may be in a steady state of growth—cell populations in log phase—or of dynamic equilibrium with constant size. The normal adult animal or plant has many cell systems of the latter type, the *renewal systems*,¹ which depend for constancy of size on a balance between birth (or intake) and death (or release). Examples are the bone marrow, the skin and its appendages, and the GI epithelia in animals and the meristems of the root and the shoot in plants.

In this paper, cell renewal systems are considered from a theoretical viewpoint. The emphasis is less on the systems as such than on the kind of information about them that can be gained from experiments. Whatever the nature of a particular cell system, the information defining it has a definite structure that depends on the number of compartments. The demonstration of this structure constitutes the first and longest part of this paper. In the second part, variability of the kinetics of single cells is briefly discussed, and in the third part, the types and variety of possible control systems are considered.

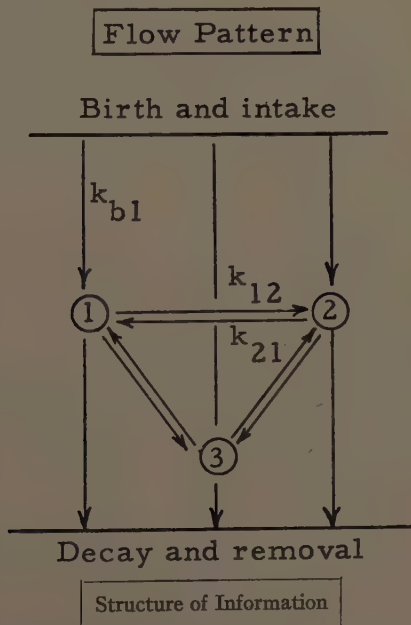
Deterministic Model of a Cell Renewal System: Structural Considerations

A formal treatment of a cell system requires that the population be partitioned into compartments or states. These may be characterized in any manner, for example, by morphology, function and location. It is not necessary to take into account all the distinctions that can be readily made; neither is it always sufficient. Ideally, the partition should be such that all the differences of consequence to the phenomena studied, and none but these, are used to determine the classification of a given element. This is easier said than done, since usually only the investigation itself will reveal whether or not the method of classification being used is appropriate. No rules exist for determining a priori which features to consider and which to disregard in any particular case. The method of classification must be adjusted to the results of an investigation in progress by dropping distinctions that turn out to be irrelevant and searching for additional criteria if the ones in use seem insufficient.

A statement of number (or weight, or some other measure) of the cells in

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each compartment constitutes the size distribution pattern of the cell system. If the total size of the system is known or equated to 1, then any one size parameter can be computed if all the others are known. Hence, the "size information" for a system with R compartments is a structure of $(R - 1)$ independent dimensions. Since any one of the R parameters can be left for computation, there are R different ways of combining $(R - 1)$ observations and 1 computation into a complete statement.



$R(R - 1)$ transition parameters, k_{ij}
 $+ 2R$ birth and decay parameters, k_{bi} and k_{id}

$R^2 + R$

$- R$ steady state conditions, $K_{in} = K_{out}$

R^2 independent dimensions of information

and: $2R$ different sets of data sufficient for assessing flow pattern of first compartment,

$2(R - 1)$ ways for second and

$R! 2^R$ different sets of data for assessing flow pattern of entire system.

FIGURE 1. Flow pattern of a steady state system.

In a steady state system, the constancy of size distribution is maintained by controlled flow of cells. In general, cells can flow from each compartment into every other; furthermore, in each compartment cells can be added to the system by birth or intake, or lost by death or removal. The complete statement of all flow rates constitutes the flow pattern of the system (FIGURE 1). A complete flow pattern includes the following components: the transition parameters, k_{ij} , for every pair of compartments ij ; the birth parameters, k_{bi} , for every compartment i ; and the decay parameters, k_{id} , for every compartment i . These parameters have the dimensions of cells per unit time (or mass per unit time, depending on the unit of size used).

If a system has R compartments, there may be as many as $(R - 1)$ distinct streams of cells going from each compartment into each of the other compartments; there are, thus, a total of $R(R - 1)$ transition parameters. Also, there may be R parameters each of birth and decay, giving a total of $R(R + 1)$ flow parameters.

If the system is in a steady state then, for each compartment, the total inflow equals the total outflow. This yields a total of R constraints that can be used to compute R parameters, and leaves R^2 parameters that must be estimated from observation. This number, R^2 , is the irreducible dimensionality of the flow pattern of the system; no flow pattern is completely known unless R^2 rates have been estimated, and no amount of knowledge concerning some of the flow rates can substitute for complete lack of knowledge concerning the others in establishing a complete flow pattern.

R^2 observations and R computations can be combined in several ways to establish a complete flow pattern. Starting with any one compartment in an R -membered system, R separate streams of cells must be accounted for which might enter the compartment from the $(R - 1)$ other compartments and from the outside (cells immigrating or produced), and also an equal number of streams that might leave the compartment. The steady state condition that the total number of cells entering must equal the total number of cells leaving allows computation of any one of the $2R$ flow rates if the other $(2R - 1)$ are known. Therefore, there are $2R$ different sets of observations, of $(2R - 1)$ parameters each, which suffice to determine the flow pattern for the first compartment. In the case of the second compartment, since the rates of the two streams of cells connecting it with the first compartment are known, $(2R - 2)$ rates remain to be determined. Of these, any one can be computed if the other $(2R - 3)$ are known, which means that there are $(2R - 2)$ ways of determining the flow pattern for the second compartment. Application of similar reasoning to the remaining compartments shows that the total number of different approaches to establishing a complete flow pattern is

$$2R(2R - 2)(2R - 4) \cdots = R!2^R.$$

The number $R!2^R$ measures the degree of experimental flexibility available in the determination of a flow pattern. The function increases rapidly with R ; its values for systems with 1, 2, 3, and 4 compartments, respectively, are 2, 8, 48, and 384. Hence, the experimental flexibility increases rapidly with the number of compartments, and this is needed. A single type of experimental study will usually furnish the values of one to a few parameters; the determination of, for example, 48 or more parameters for a single system (with 3 or more compartments) is a major undertaking that would hardly ever succeed without great flexibility of possible approaches.

In practice, the required number of parameters in any particular system is hardly ever determined by direct observation. Usually assumptions based on general knowledge are used to achieve vast reductions in the number of flow parameters. The assumption most frequently used is that of an orderly flow, which implies (1) that a proliferating cell goes through the various phases of mitosis, postmitotic gap (G_1), DNA synthesis (S), and premitotic gap (G_2) in the order given, (2) that a differentiating cell goes through the various stages

of maturation in a regular sequence without shortcuts and reversals (dedifferentiation), and (3) that both cell production and cell decay are restricted to one or a few compartments. These assumptions are often justified, at least in first approximation, but they must be made explicit and used with caution. As an illustration, consider the situation in erythropoiesis (FIGURE 2). The earliest recognizable erythroid cell, the erythroblast, can come from another erythroblast or from a more primitive cell out of the "primitive progenitor pool."² The erythroblast can differentiate into a pro-normoblast which, in turn, can multiply or lead into the normoblast series. The earlier members of this series can multiply; the later ones differentiate directly through the nucleated reticulocyte into the mature erythrocyte. Recent studies have indicated, however, that this simple flow pattern may be insufficient to deal with problems of control of cell production; Alpen and Cranmore³ postulated a shunt leading directly from the erythroblast to the erythrocyte to explain how an

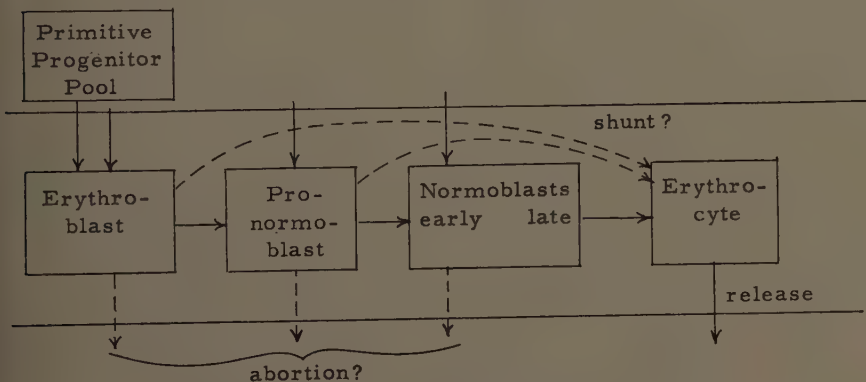


FIGURE 2. Bone marrow, erythroid system: flow pattern.

overgrown erythropoietic system returns to its normal size, and Stohlman⁴ found indications of cell death (abortion) occurring in the proliferative compartments and suggested that the proliferation rate was regulated by control of the death rate. This example indicates some limitations of the assumption of orderly flow.

Even more caution is needed in dealing with systems that have been subjected to some violent perturbation, because they may contain cell types that normally do not occur. For instance, treatment with X rays or various poisons will stop many premitotic (tetraploid) cells from entering mitosis; after the block subsides, many of these cells, instead of going directly into mitosis, again go through the stage of DNA synthesis and thus become octoploid before mitosis. The blocked cell must combine features normally found in cells in different stages, G_1 and G_2 . Another example is the acidophilic normoblast, a cell with the nucleus of a normoblast and cytoplasm like that of a fully mature erythrocyte, which was found by Lamerton *et al.*⁵ in large numbers in rat bone marrow after X irradiation.

In a steady state system, size and flow parameters are related to time parameters. If n_i is the number of cells in the i 'th compartment, and K_i the num-

ber of cells entering or leaving the compartment per unit time, in any direction (absolute flow rate), then

$$n_i/K_i = t_i$$

where t_i is the *turnover time*,¹ or the time required for entry (or removal) of a number of cells equal to the total number of cells contained in the compartment. This relation can be used to determine either n or K , if the other and the turnover time are known. If all or part of a steady state system is traversed by a single stream of cells in a regular order, then a single flow rate obtains which equals the ratio of number of cells n over turnover time t for every compartment:

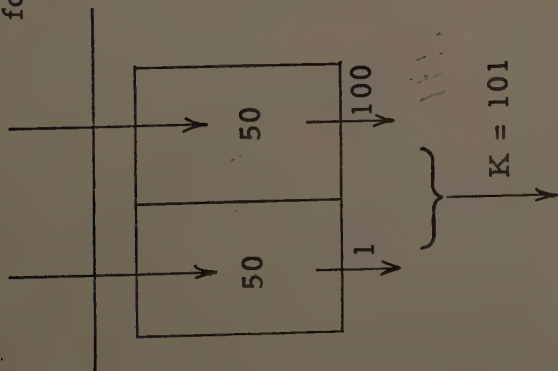
$$n_i/t_i = K.$$

This relation is often used to compute the generation time from mitotic index and mitosis time, or from labeling index synthesis time. The relation will not hold for cell populations in logarithmic growth,⁶ but it will hold for renewal systems if cells are withdrawn from the proliferative compartment just before or after mitosis. This was found to be the case for the intestinal epithelium.⁷

The reciprocal of the turnover time is the turnover constant (in cells per cell per unit time). It has the nature of a relative flow rate or, in the absence of other types of transitions, of a specific birth or decay rate. The turnover time, itself, is not a "natural" measure of anything; it is sometimes equated to the more interesting parameters, mean time of sojourn or (in a proliferative compartment) generation time; however, its relation to either of these is not necessarily simple. FIGURE 3 represents a hypothetical case in which an entity considered as a single compartment actually contains two kinds of cells with sojourn times of 50 days and 0.5 day, respectively. If the compartment contains 50 cells of each kind, the total flow through it will be 101 cells/day, 1 cell being one kind and 100 the other. If the mean sojourn time is determined by averaging a sample of cells entering the compartment during a given interval, it is found to be 1.01 days; averaging a sample of cells present in the compartment at a given moment gives 25.25 days. Either average can be useful, depending on the situation; neither corresponds to the actual sojourn time of either kind of cell.

The situation diagrammed in FIGURE 3 is admittedly extreme, but not necessarily unrealistic considering that there may be two kinds of lymphocytes that look alike but have vastly differing life spans.^{8,9} In one common situation, a moderate amount of hidden inhomogeneity is the rule; this is the case of differentiation following division (FIGURE 4). In granulopoiesis, for example, the most differentiated cell known to divide is the myelocyte, and the least differentiated cell that does not divide any more is the metamyelocyte. Patt and Maloney¹⁰ labeled the DNA of dividing cells in the dog and measured the elapsed time between labeling and appearance of the first labeled metamyelocytes. This was found to be about 16 hours. The postsynthetic (= premitotic) gap, G_2 , together with the mitosis time lasts about 5 hours; hence there must be a time of about 11 hours during which a cell resembles a myelocyte but is actually differentiating into a metamyelocyte. A similar situation

Mean Time of Sojourn (Transit Time)
for inhomogeneous populations



a - sampling over a cohort of incoming
(or outgoing) cells:

$$T_a = \frac{1}{101} \cdot 50 + \frac{100}{101} \cdot \frac{1}{2} = 1.01 = \text{turnover time}$$

b - sampling over cells present in compartment:

$$T_b = \frac{50}{100} \cdot 50 + \frac{50}{100} \cdot \frac{1}{2} = 25.25$$

FIGURE 3. Transit time estimates.

obtains in the intestinal epithelium, where proliferating cells are located in the crypt and mature cells on the villi. It takes about 10 hours for DNA-labeled cells to reach the villus. Of this interval about 6 hours may be spent in finishing the synthesis phase and going through the premitotic gap and mitosis; the remaining time must be taken up with differentiation, which indicates that the crypt population contains a fair share of cells that look like proliferative cells but actually will not divide any more.⁷ In this instance, it was possible to differentiate between proliferating and differentiating cells by using the electron microscope: proliferative and mature cells have characteristically different endoplasmic reticula, and cells containing elements of both types were found in the location presumably occupied by differentiating cells.¹¹ In such situa-

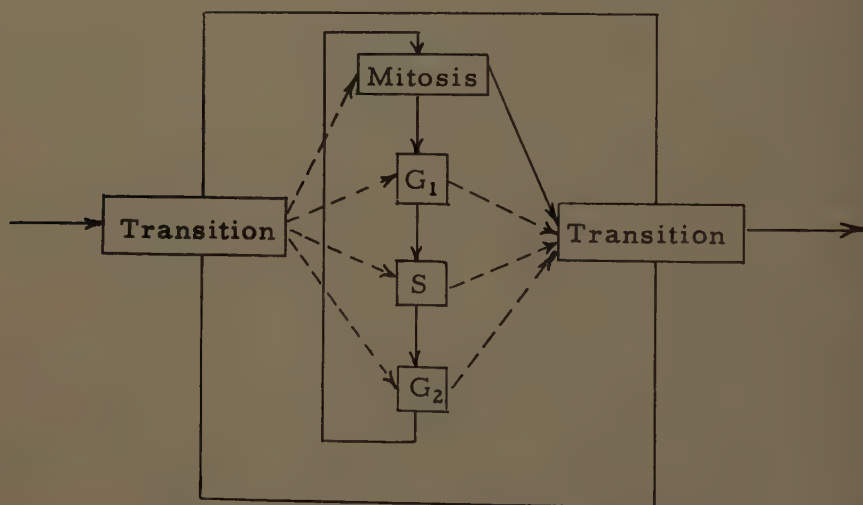


FIGURE 4. Fine structure of proliferative compartments.

tions, failure to take into account the presence of two kinds of cells will result in falsely equating turnover time to the (shorter) generation time.

Stochastic Kinetics

All living structures last for varying lengths of time. In any natural population some of the variation may be due to differences in genetic endowment and environment, but even when both these factors are kept as uniform as possible, differences in lifetime remain. This is true for pure clones of microorganisms (T. W. James, elsewhere in this monograph) as well as for higher organisms.¹²⁻¹⁴ Various observations of movements of labeled cells have demonstrated inherent variabilities in the duration of lifetime of mature cells. A direct study of the variations in the duration of the proliferative cycle and its various components has been made possible by the recent development of the technique of specific labeling of DNA combined with high resolution autoradiography.

In a microscopic study of a proliferative cell population, attention can be restricted to cells in mitosis or, better still, in some particular phase of mitosis,

that is, to a subpopulation of cells synchronous at the time of sacrifice, within the limits determined by the duration of the phase considered. If the tissue studied has been treated with labeled thymidine during a short period at some time preceding sacrifice, then the cells that were in synthesis phase at that time will be labeled. By sacrificing at increasing intervals after labeling, one can reconstruct the alternation between synthesis and nonsynthesis phases preceding the mitosis in process at the time of sacrifice, with an accuracy initially depending on the duration of mitosis (or some portion of it) and the length of time during which label was available (FIGURE 5). Specifically, if the interval between labeling and sacrifice was short (1 hour or less) then the cells in mitosis

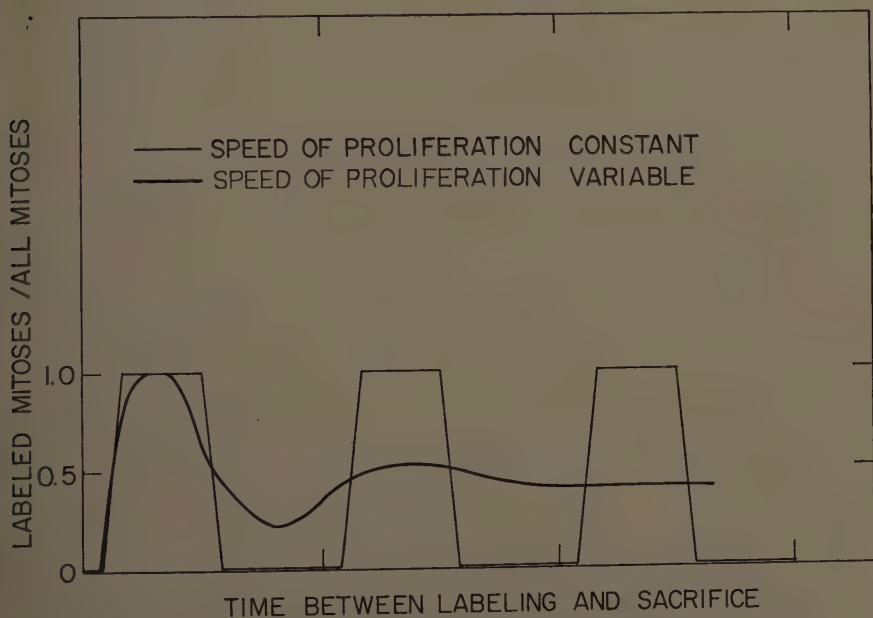


FIGURE 5. Time course of fraction-labeled mitoses (Schema by D. E. Wimber).

at sacrifice will have been in an earlier stage of mitosis or in the premitotic gap, G_2 , at the time of labeling and will therefore be unlabeled. With longer intervals (several hours) cells in mitosis at sacrifice will have been in synthesis phases at labeling, and will be labeled. As the interval is increased, cells in mitosis at sacrifice will not be labeled because at the time of labeling they were in one of the nonsynthetic phases (G_1 , mitosis, G_2) preceding the last S-phase. With a further increase in interval, labeling will be seen due to the S-phase once removed from the mitosis in progress at sacrifice, and this progress will continue. If all cells were to go through the proliferative cycle at the same speed, then cells synchronous at sacrifice would have been synchronous at any previous time; in this case, the curve of "labeled mitoses/all mitoses" versus "time between labeling and sacrifice" should show a regular alternation between periods when all mitoses are labeled and periods when none is labeled.

The curves actually found followed this "constant-speed" curve only along the first portion, showing a fairly fast rise from zero to a high value of "fraction mitoses labeled," followed by a slower descent which did not reach zero; the second wave, when noticeable at all, was much less pronounced than the first. The interval taken for the first transition (from zero to about 100 per cent labeling) represents the combined duration of mitosis plus G_2 , plus variations in the duration of these two phases, plus availability of label; the interval during which labeling is high is the duration of the S -phase; the transition from high to low is slower than the preceding transition from low to high because of the added variability of the duration of the synthesis phase. The accurate estimation of the variability in duration of the synthesis phase would involve setting up a convolution integral taken over all observed durations of [mitosis + G_2 + S'] and all ways in which this duration can be split up into two components. Data at hand are not remotely sufficient for such treatment. Therefore, an

TABLE 1
MEANS AND STANDARD DEVIATION OF DURATIONS OF PROLIFERATIVE PHASES
(In Hours)

Phase	Intestinal epithelium		Tradescantia root	
	Mean duration	Standard deviation	Mean duration	Standard deviation
Synthesis, S	7½	1½	10.8	1.7
Premitotic				
Gap, G_2			2.7	0.8
Prophase	2	?	1.6	
Metaphase			0.3	
Ana-telophase			0.6	3.7
Postmitotic				
Gap, G_1	9½	3¾	4.0	

estimate of the variability was obtained under the assumption (which is not too far-fetched) that the durations of succeeding phases are independent. Similarly, the ascent of the next wave has a slope that results from variations of a whole generation time plus one mitosis and G_2 phase. The aggregate variability is large enough to make the second wave much less pronounced than the first. The contributions to this variability from all components except G_1 can be estimated from the analysis of the first wave of high labeling indices, assuming, again, independence of duration of successive phases.

The variability of duration of the various phases of the generative cycle has been analysed in this way in two cell systems, the intestinal epithelium of the mouse⁷ and the root tip of *Tradescantia*.¹⁵ The results (TABLE 1) indicate that the largest contribution to the variability of proliferation times is due to the postmitotic gap G_1 .

There are indications that the G_1 period is responsible for differences in generation times not only within but also between cell populations. The generation times in the hair matrix and the intestinal epithelium of the mouse relate about as 2:3; the difference was found to be due chiefly to differences in the

duration of G_1 .¹⁶ M. Mendelsohn (article in press) found that in a series of mammary tumors in mice, with generation times varying from 1 to 3 days, the duration of G_2 and S varied little, which again indicates that the variations are due to variations in duration of G_1 . Investigations on tissues growing at slow rates and at maximum rates will show whether the strong dependency of proliferation times on the duration of G_1 is a general rule.

Synchronicity of cells in rapidly growing tissues thus tends to disappear within one or two generation cycles, which results in pronounced randomness of stage within a proliferating population. The randomness is counteracted, to some degree, by synchronizing effects of diurnal variations, but these appear to be of limited scope and restricted to slowly growing tissues; they disappear in the presence of a strong proliferative stimulus, for example, in the case of liver regeneration following partial hepatectomy.

The pronounced variability in the behavior of individual cells suggests that stochastic features are characteristic of cell populations and may well have some basic function. However, merely to obtain a description of fluctuations by measurements is much more difficult than the estimation of the mean values that make up the description of the deterministic kinetics. As long as major difficulties remain in establishing a fair number of descriptions in terms of mean values, there is not much hope for rapid progress in the analysis of fluctuations by direct studies of cell populations.

Controls in Steady State Cell Populations

The regularity—although limited—in the behavior of isolated cells suggests the presence of internal controls, a topic discussed in this monograph by D. Mazia. The existence of a steady state in the undoubted presence of perturbations suggests external controls, that is, actions on various transitions in a system produced by agents outside a particular cell involved in a transition process. Only external controls will be discussed here, and the aspects considered will be the range of types of controls known and the variability of available control systems.

Types of external control. T. T. Puck (article in press) has pointed out that randomization of phase is a simple and very powerful means of preserving a steady state, and that it has considerable survival value. The sensitivity of cells to various external agents depends, to a greater or lesser degree, on the phase the cells are in; sensitivity to X rays is an example. In a cell population with randomized phase, the proportion of cells in a sensitive state at any given time will fluctuate within narrow limits; therefore, the sensitivity of the system to any kind of perturbation will tend to remain constant. It is possible that some systems, or parts of systems, remain in steady state solely through randomization of duration of stages. Of course, no such system can preserve a steady state indefinitely; however, the probability of failure may be small compared to the life span of the organism.

Specific controls are best known for birth and decay processes, less known for other transitions. Specific stimulation of production is known in several systems. The existence of one or possibly several substances that stimulate red-cell production (erythropoietin) is well established although the mode of action

is still doubtful.¹⁷ The existence of leukopoietins and thrombopoietins has been suspected.¹⁷ Sex hormones have stimulating effects on growth of various systems; some of the regulatory effects within the endocrine system operate on the kinetics of the cells involved.

Specific suppressors of cell production are well known from embryological studies;¹⁸ in the adult mammal, they seem to prevent growth of the liver (A. D. Glinos, elsewhere in this monograph). Osgood¹⁹ has proposed a unifying concept of growth regulation by specific suppressors that fits a considerable range of situations. Specific suppression of cell death is, in principle, as effective a control of size as is suppression of cell birth; Stohlman⁴ has proposed a model of the regulation of erythroid elements by this mechanism.

Range of possible control systems. If a steady state system is subjected to a serious perturbation, then the size distribution pattern will be altered. A return to the standard pattern depends on controlled alteration of flow parameters. However, it is not necessary to control every single transition rate in order to restore an altered size distribution pattern. Each particular control system may thus be based on a pattern of control points that does not necessarily embrace all the components of the flow pattern. Each controlled rate, in addition, may be affected in a number of ways: control may be exercised by randomization, by specific stimulation of cell production, of cell death, and other transitions as well, or by specific suppression of cell production and death and, presumably, other transitions. There is no reason to believe that each type of control operates in a unique fashion. D. Mazia has discussed elsewhere in this monograph the variety of available control points within a cell, and it may be assumed that more than one of them are actually used.

One aspect of the variety of control systems can be treated by the type of analysis used in the first section of this paper. This is the variety of control point patterns. Consider a given compartment in an R -membered system. It has been stated that there may be as many as $2R$ separate streams of cells entering or leaving the compartment, and that the flow rate of any one stream can be computed provided the system is in a steady state and the other $(2R - 1)$ flow rates are known. This implies that the compartment can be kept in a steady state by operating on a single flow parameter, provided, of course, that the disturbances to be checked are within the range of control of the particular parameter. In the simplest case, a one-compartment system, there are two streams of cells—in and out—and control of either one is sufficient to keep the system in a steady state (which does not exclude the possibility that both streams are actually controlled); in other words, a single compartment may be birth-controlled or death-controlled, or both. A two-compartment system has 6 possible streams of cells, and at least 1 for each compartment must be controlled; there are 4 possible controls for the first compartment and 2 for the second, a total of 8 different patterns of control points. In general, it is seen that there must be at least R control points from which a steady state system may be held in balance, and that the number of ways of selecting a minimal pattern of control points is precisely equal to the number of different experimental approaches available for determination of a flow pattern, namely, $R!2^R$ (FIGURE 1).

If some transitions do not occur, as in systems with orderly flow, then the range of possible control point patterns will be accordingly reduced. However, all the caution required in using simplifying assumptions should be applied in considering possible control systems. For example, a rate of dedifferentiation that is normally negligible may reach significant values in a depleted system calling for maximum cell production.

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ENVIRONMENTAL FEEDBACK CONTROL OF CELL DIVISION

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Environmental feedback systems contributing to the homeostatic control of cell division would seem to require the following two relationships between a given cell population and its environment: (1) dependence of the rate of metabolic reactions involved in cellular growth and division on the concentration of some specific constituents of the extracellular environment; and (2) dependence of the extracellular concentration of these constituents on the metabolic activity of the cells. Evidence for the existence of these relationships in two cases of physiological significance for the control of cell division in the mammalian organisms will now be presented.

The first case concerns the control of cell division during the regeneration of the mammalian liver following partial hepatectomy. Tissue culture studies¹ demonstrated a comparable outgrowth in a high concentration of serum from partially hepatectomized rats and in a low concentration of normal serum. A high concentration of normal serum showed inhibitory effects. These findings were considered to indicate an inverse relationship between the concentration of certain serum constituents and the rate of cell division in the liver. In order to test the validity of this concept two obvious consequences were put to test. If liver cell proliferation is controlled by the concentration of certain growth-inhibitory constituents of the serum, it should be possible to induce cell division in the intact resting liver by lowering the concentration of serum constituents. Conversely by increasing the concentration of serum constituents it should be possible to inhibit cell division in the regenerating liver of partially hepatectomized animals. Plasmapheresis and fluid intake restriction were used respectively in order to test these possibilities.

In the plasmapheresis experiments blood corresponding to 25 per cent of the initial total blood volume of the animal was withdrawn every 6 hours. The bleedings were followed by reinjection of the blood cells suspended in an equal volume of physiological saline. Under these conditions cell division was induced in the resting liver of adult rats, and was intensified with increasing duration of the treatment (TABLE 1). In the fluid intake restriction experiments two groups of rats were used differing with regard to the extent of the partial hepatectomy. All animals were partially hepatectomized and tube-fed an identical isocaloric diet containing 3 per cent water. The controls were given drinking water ad libitum but the experimental animals were deprived of drinking water for the duration of the experiment which was 64 hours, starting 16 hours prior to the operation and continuing for 48 hours postoperatively, at which time the animals were sacrificed. Total body water loss under these conditions was within 10 per cent of the body weight confirming the finding that no appreciable metabolic disturbance occurs in the rat after 3 days of water intake restriction (A. V. Wolf and F. C. Meroney, unpublished data). In both the experimental groups, an effective inhibition of cell division was observed, the mitotic index decreasing by 80 per cent in the first group and by 99 per cent in the second (TABLE 2).

The evidence provided by these experiments indicates that rates along the reaction chain leading to liver cell division depend, at some key links at least, on the concentration of certain constituents of the serum. The reasons that lead us to think that one of these links concerns a possible relationship between plasma protein synthesis and liver cell protein synthesis have been discussed in detail elsewhere.² According to this concept the amount of extracellular plasma protein synthesized by the individual liver cell per unit time would be related within certain limits to the amount of intracellular protein present, by a constant ratio. Lowering of the level of the extracellular protein beyond a certain limit would result not only in increase of extracellular plasma protein

TABLE 1
INDUCTION OF CELL DIVISION IN THE RESTING LIVER BY PLASMAPHERESIS*

Treatment	60 Hours		96 Hours	
	Number of animals	Liver mitotic index per cent	Number of animals	Liver mitotic index per cent
Control	6	0.002	6	0.004
Plasmapheresis	8	0.040	6	0.225

Induction of cell division in the resting liver by plasmapheresis.

* In the control group the blood was merely withdrawn and reinjected with the animals submitted to the same stressful conditions as in the experimental group.

TABLE 2
INHIBITION OF CELL DIVISION IN THE REGENERATING LIVER
BY FLUID INTAKE RESTRICTION*

Extent of hepatectomy	Treatment	Number of rats	Liver mitotic index per cent
30% (Median lobe)	Controls	10	0.162
	Fluid restriction	9	0.036
10% (Caudate lobe)	Controls	8	0.033
	Fluid restriction	8	0.002

* Redrawn from Glinos.²

synthesis but also in activation of the synthesis of intracellular protein and cytoplasmic RNA. This in turn would induce synthesis of nuclear RNA and DNA followed by cell division. In turn the lowered plasma protein level would be restored as a result of the increased number of liver cells producing extracellular protein. Thus the level of extracellular plasma protein would control the rate of synthesis of intracellular protein and ultimately the total number of cells in the liver, the extracellular protein level itself depending on the number of liver cells present. The resulting environmental feedback system controlling liver cell division is shown schematically in FIGURE 1.

According to this postulate, the activation of the synthetic reactions preceding cell division in the liver should proceed from the cytoplasm of the liver cells to the nucleus. Since this activation is attributed to a decrease of the extracellular protein concentration, which in the liver is expected to proceed

from the periphery to the center of the lobule,² this same order should be apparent also in the activation of the individual cells of each lobule. These characteristic features of cell division in the liver should be present both during regeneration after partial hepatectomy and after plasma protein depletion. Finally the restoration of normal extracellular protein concentrations should deactivate the cells and inhibit mitosis in the regenerating liver.

The available evidence from relevant observations is consistent with these propositions. Following partial hepatectomy, there is an initial period of approximately 24 hours during which no cell division occurs. Cellular growth is detectable both morphologically by the increased cytoplasmic, nucleolar and nuclear volumes, and functionally by the activation of synthesis of the various cellular constituents. The latter is manifested by an increase of the rate of incorporation of labeled precursors. As a result of increased synthesis the net amounts of the various cellular constituents increase. The time sequence of the activation of synthesis and net increase of the cellular proteins and nucleic acids as it emerges from the extensive work of numerous investigators³⁻¹³ clearly indicates that cytoplasmic RNA and protein precede nuclear RNA and DNA. The activation of cytoplasmic RNA and protein synthesis can also be detected

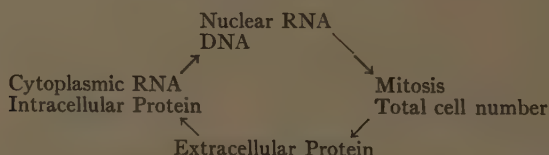


FIGURE 1. See text. Reproduced by permission from the Johns Hopkins Press.²

histochemically through the disappearance of the basophilic ribonucleoprotein associated bodies, from the cytoplasm of the activated cells that then stain uniformly with the dye.² The sequential analysis of these changes as they occur after partial hepatectomy shows that the cells in the periphery of the lobule are activated within 30 min. after the operation (FIGURE 2) and that the changes proceed gradually toward the center reaching the cells adjacent to the central vein 8 hours later.^{2,14} The relationship of these changes with cell division was strikingly demonstrated because confirming an earlier observation by Harkness,¹⁵ we found that mitosis that began 24 hours after the operation started also in the periphery and gradually expanded toward the center of the lobule. Thus the characteristic features of the activation of the liver cells after partial hepatectomy seem to conform to the predicted pattern.

The liver cells are also activated by plasma protein depletion. Following a sudden decrease of the concentration of serum proteins the rapid occurrence of changes in cytoplasmic ribonucleoprotein identical with the changes observed after partial hepatectomy denotes the early activation of the cells in the periphery of the liver lobules (TABLE 3). It appears that this activation occurs when the level of serum proteins is lowered by more than 12 per cent. Since it is known that the main site of serum protein synthesis is the cytoplasm of the liver cells,^{16,17} lowering the level of serum proteins would be expected to activate cytoplasmic syntheses. However, as it has been shown previously the

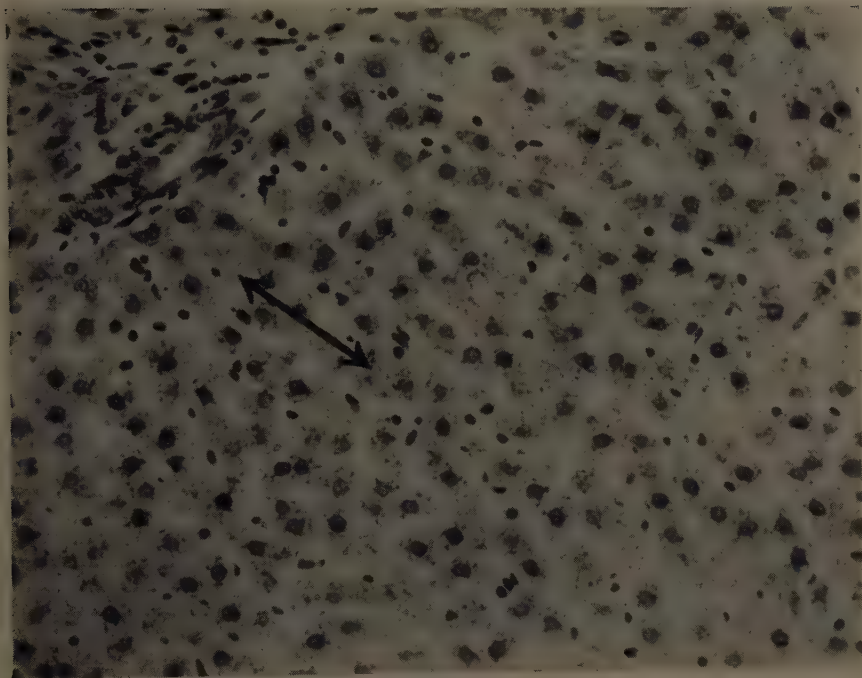


FIGURE 2. Activation of liver cells in the periphery of the lobule 30 min. after partial hepatectomy. Upper arrowhead points to peripheral cells with uniform basophilia of the cytoplasm. Lower arrowhead points to centrally located cells with cytoplasm containing coarse ribonucleoprotein-associated basophilic bodies. Reproduced by permission from the Johns Hopkins Press.²

TABLE 3
ACTIVATION OF LIVER CELLS IN THE PERIPHERY OF THE
LOBULE BY PLASMA PROTEIN DILUTION*

Treatment	Fluid	Serum protein change	Liver ribonucleo-protein change
Addition	Saline	-11.8	0
	Dextran	-31.2	+
	Serum	+7.9	0
Replacement	Saline	-19.2	+
	Dextran	-37.8	+
	Serum	-8.7	0

* A total of 6 male adult rats was used. Addition refers to a single intravenous injection of 5.5 ml. of fluid. Replacement refers to a 5.5 ml. single plasmapheresis treatment. All animals were sacrificed 2 hours after treatment. Serum protein change refers to the per cent difference between the values obtained before treatment and those obtained by sacrifice. Liver ribonucleoprotein change refers to the disappearance of the basophilic bodies from the cytoplasm of the cells in the periportal area. Reproduced by permission of the Johns Hopkins Press.²

activation induced by prolonged plasmapheresis is not limited to the cytoplasm but extends also to the nucleus, the liver cells finally undergoing mitosis, precisely as in the response following partial hepatectomy. Confirmation of this finding was recently obtained by Naora and Naora¹⁸ who demonstrated increased incorporation of P³² into the DNA of the liver of normal adult rats submitted to plasmapheresis.

The type of nucleocytoplasmic interactions involved in the premitotic activation of liver cells can then be defined as retrograde, in contradistinction to the large body of information pertaining to nuclear control of cytoplasmic function. The general significance of such retrograde nucleocytoplasmic interactions for the homeostatic control of cellular growth and division has been discussed recently elsewhere.¹⁹

On the basis of the foregoing it would then seem reasonable to state that the characteristic features of cell division in the liver during regeneration after partial hepatectomy and after plasma protein depletion are identical.

The last consequence of the postulate regarding the environmental feedback system controlling liver cell division is that the restoration of normal extracellular protein concentrations should deactivate the cells and inhibit mitosis in the regenerating liver.

As we have already seen, the restriction of fluid intake of partially hepatectomized rats effectively inhibits liver cell division. The concentration of serum proteins was increased in these experiments²⁰ but there is no direct evidence implicating the serum proteins in the mitotic inhibition obtained. Although, for reasons, some of which were discussed elsewhere,² intravenous infusions of normal serum do not affect mitosis in the regenerating liver,²⁰⁻²² the existence of serum constituents capable of inhibiting liver cell division was confirmed by a number of experiments involving intraperitoneal injections of normal serum into partially hepatectomized rats (H. F. Stich, elsewhere in these pages).²³ In these experiments also, no clues have been obtained concerning the identity of the constituents of the serum that control liver cell division. This question is now under investigation in our laboratory and as these experiments are carried out, the necessity of considering and carefully controlling the nutritional and circulatory conditions following partial hepatectomy is becoming evident once more;^{2,4} failure to recognize this necessity may well account for the reported inconsistencies regarding effects of injections of serum from partially hepatectomized animals (H. F. Stich, elsewhere in these pages).^{2,22-26} A striking illustration of this point is provided by our recent finding that under certain nutritional and circulatory conditions even normal serum may stimulate liver cell division by increasing the supply of essential metabolites to the liver (TABLE 4).

The second case of environmental feedback control of cell division concerns the postnatal development of the mammalian organism. In this area of sparse knowledge, the progressive decline of the rate of cell division in the course of development, represents perhaps the only important generalization that can be made at the present. It is supported by a large number of early as well as recent observations on the growth of normal and regenerating tissues at various developmental stages of many species including mammals.²⁷⁻³⁷ On the basis of this generalization the major descriptive stages of mammalian postnatal de-

velopment can be defined rationally. Thus if we divide development into three main stages, growth, maturity, and senescence, the first would be characterized by cell division rates exceeding cell replacement needs, the second by division rates equalling replacement needs, and the third by cell division rates inadequate for the replacement of the cells of some of the most important tissues of the body. This statement is by necessity an oversimplification; nevertheless it has the merit of transforming the formidable problem of postnatal development into a series of questions amenable to experimental probing. One of these questions is whether the decline of the rate of cell division during development is endogenous, arising from progressive structural and functional alterations within the cell or whether it is exogenous, arising from changes in the extracellular environment. Although hypothetical answers along either one of these

TABLE 4
STIMULATION OF LIVER CELL DIVISION IN STARVED RATS BY NORMAL SERUM*

No. of mitoses/50,000 cells			
Starvation		Starvation + serum I.P.	
Rat no.		Rat no.	
1	5	7	5
2	2	8	4
3	1	9	70
4	2	10	36
5	0	11	5
6	2	12	13
Mean	2	Mean	20

* Male Walter Reed pathogen-free rats weighing from 400 to 450 gm. were used. Numbers 1 to 6 were submitted to a median lobe partial hepatectomy, given water ad libitum but no food, and sacrificed 30 hours later. The same procedure was followed with rats No. 7 to 13 with the difference that these rats were given 3.5 ml. of pooled normal rat serum intraperitoneally immediately after the operation.

two alternatives have formed the basis of discussions concerning aging, radiation injury, and carcinogenesis,^{38,39} experimental evidence bearing directly on the question is meager. The most well-known work relevant to this question, is undoubtedly represented by Carrel's and Ebeling's early tissue culture experiments suggesting that during development there occurs a progressive accumulation of growth inhibitory factors in the serum.⁴⁰

The tissue culture area measurements used by these early investigators to evaluate cell proliferation are open to serious criticism and prompted by some suggestive findings obtained during the course of other work⁴¹ a reinvestigation of this question was begun recently in our laboratory. In these experiments nuclear counts are used to evaluate the rate of cell division in replicate cultures of the L strain, containing sera from rats of various ages. Cellular proliferation is expressed in terms of the factorial increase of the initial population after incubation of the cultures for four days. FIGURE 3 shows the results obtained in 56 experiments using sera from rats varying in age from 4 to 40 weeks. Between these ages the rate of growth of the animals undergoes considerable change as shown in FIGURE 4. Consideration of the tissue culture findings in

conjunction with the data concerning the growth of the animals from which the serum was obtained, indicates that the growth-promoting activity of the serum is inversely related to the growth rate of the animals. Thus during the period of the most rapid growth of the animals, as between four and six weeks of age, the activity of the serum reaches its lowest value. Later, as the growth of the animals becomes progressively slower, the activity of the serum gradually increases.

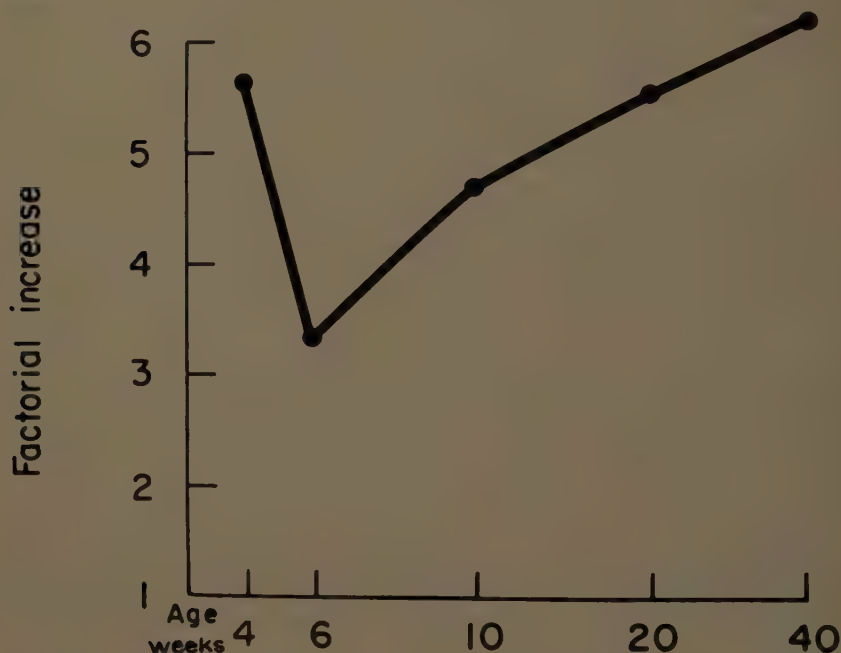


FIGURE 3. Growth of the L strain in sera from rats of different ages. Five groups of Walter Reed pathogen-free rats varying in age from 4 to 40 weeks were used as serum donors. From each age group several pools of sera were obtained and used together with Earle's balanced saline as the sole medium in setting up replicate cultures of the L strain. A total of 56 such experiments were performed. Each point of the curve represents the mean factorial increase of the L-strain population after 4 days of growth in the sera of the respective age group. With the exception of the 20 week age group, differences between consecutive points are significant at the 0.01 level.

This type of kinetics, together with the fact that the activity of the youngest animals so far investigated is high, suggests that the sera of newborn and very young individuals contain rather large amounts of some unidentified growth-promoting factors; that during the early rapid growth of the individual these metabolites are utilized at a rate exceeding their rate of supply and therefore their concentration in the serum tends to decrease; and that at more advanced ages as the rate of growth of the individual decreases, the opposite situation prevails causing these metabolites to accumulate anew in the serum.

Pending extension of these findings to rats beyond 40 weeks of age, and identification of the metabolites involved through serum fractionation procedures,

the data up to date would seem to favor the hypothesis that the process responsible for the decline of the rate of cell division at least during the early stages of postnatal development, is endogenous. Changes in the cellular environment would tend not to enhance but to oppose this decline.

A consequence of this concept, amenable to experimental verification is that cells of animals at different developmental stages should exhibit a greater de-

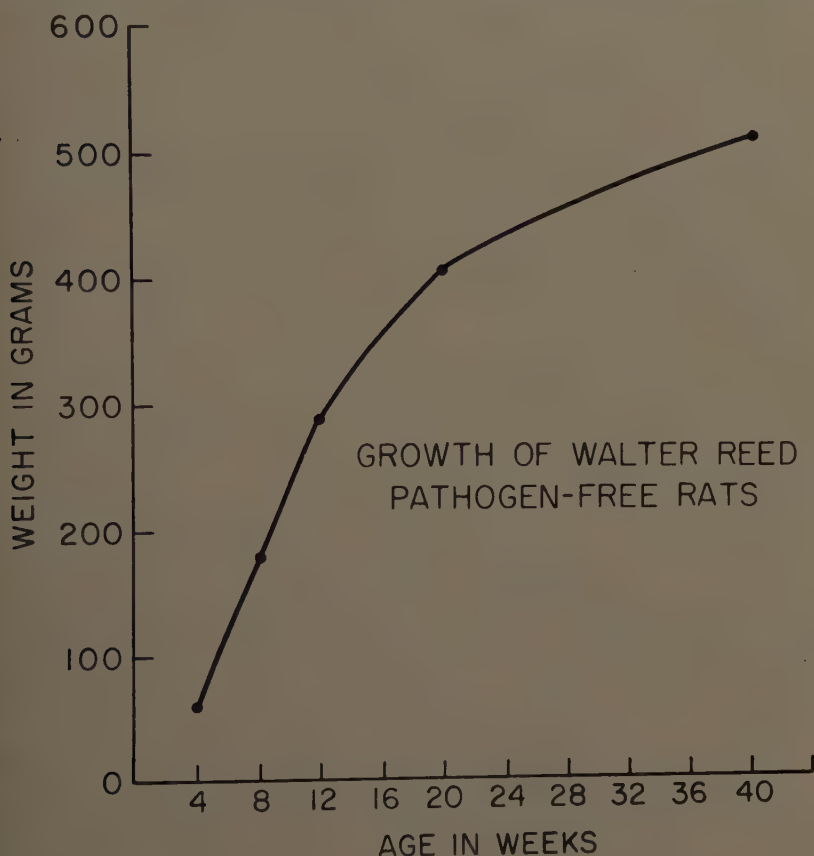


FIGURE 4.

cline of their division rate when grown in an identical environment *in vitro* than when proliferating in their own environment *in vivo*. Data from relevant observations are consistent with this hypothesis. Thus, if the percentage of livers explanted from rats of varying ages and showing outgrowth *in vitro*, is considered an indication of cellular proliferation, liver cells from rats 4 to 8 months of age show a decline of 55 per cent when compared with cells from rats 4 to 8 weeks of age, and this decline reaches 92 per cent with cells from rats 18 to 24 months of age (TABLE 5).³⁵ This is in marked contrast to liver cell proliferation *in vivo*, as the rate of the restoration of the original number of

liver cell nuclei after partial hepatectomy, exhibits a decline of only 23 per cent in rats 4 to 8 months old and of 34 per cent in rats 21 to 30 months of age (FIGURE 5).³⁴

TABLE 5
GROWTH OF THE LIVER *IN VITRO* AT DIFFERENT AGES*

Age of donor animals	Livers explanted	Livers showing outgrowth	Percentage of livers showing outgrowth	Latent period in hours
4-8 weeks	10	10	100	58
4-8 months	11	5	45	82
18-24 months	12	1	8	96

* On the basis of the number of primary liver explants showing outgrowth *in vitro*, rats 4 to 8 months of age are lagging behind animals 4 to 8 weeks of age, by 55 per cent, and rats 18 to 24 months of age, are lagging behind by 92 per cent. Redrawn from Glinos and Bartlett.³⁵

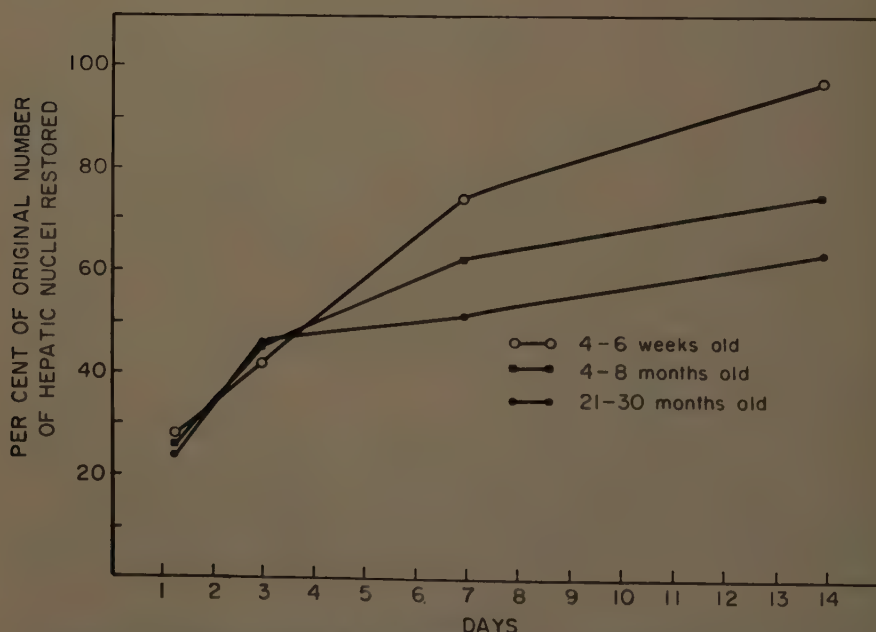


FIGURE 5. Growth of the liver *in vivo* at different ages. During the first three days after partial hepatectomy there is no difference in the rate of increase of hepatic nuclei among rats varying in age from 4 weeks to 30 months. Maximum differences are observed at 14 days when animals 4 to 8 months old lag behind animals 4 to 6 weeks old, by 23 per cent, and rats 21 to 30 weeks, lag behind by 34 per cent. Redrawn from Bucher and Glinos.³⁴

In conclusion it may be said that our findings up to date indicate that the rate of cell division during early mammalian postnatal development is due to intracellular processes. As a result, the concentration of some growth-promoting metabolites in the extracellular environment increases and in turn this tends to counteract the decline of the rate of cell division. A certain degree of homeo-

stasis of cell division is thus achieved through the operation of an environmental feedback system exhibiting the typical features described in the introduction of this paper.

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REGULATION OF MITOTIC RATE IN MAMMALIAN ORGANISMS

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Organ-specific mitotic rates, increased rates of cell division during regeneration and compensatory growth, and the cessation of mitosis in mature organs all indicate the presence of an efficient system controlling mitosis in an organism. The complexity of this system has been indicated by earlier studies. For example, nutritional conditions,¹ energy rich substances²⁻⁴ (S. Gelfant, elsewhere in these pages), hormones,^{5,6} and vitamins⁷ have been shown to influence cell division. More recently attention has been given to the interaction between specific tissues and factors of unknown chemical composition derived from these tissues as an important aspect in the regulation of cell division. The presence of mitosis inhibitors⁸⁻¹⁰ and mitosis stimulators¹¹⁻¹⁴ in different mammalian organs has been postulated and a hypothesis of autoregulation of tissue growth^{15,16} and cell growth¹⁷ formulated. It appeared of interest to apply this concept of growth regulators to the apparently unlimited growth of neoplasms as well as to the proliferation induced in various organs^{18,19} as one of the many systemic effects of tumors.²⁰

Mitotic Inhibition

The retarded outgrowth of liver explants due to the application of serum from adult rats led to the postulation of mitotic inhibitor(s).⁸ The suppression of mitosis by fluid restriction and its induction by plasmapheresis are consistent with this assumption.⁹ The partial inhibition of the mitotic rate of regenerating liver by injections of liver homogenates and serum from adult rats can also be quoted in support of this hypothesis.¹⁰ However, experiments with intact animals are affected by variations of the physiological conditions of the donors and recipients, thus leading to great variability in the results obtained by apparently equal experimental procedures. For example, injections of crude tissue homogenates can change the water level and the hormone balance and will introduce a variable amount of nucleic acid and protein precursors into the recipient. All these factors, under certain conditions, have a stimulating effect on mitosis that can easily obscure the presence of mitosis inhibitors. The effect of injections of liver homogenates on regenerating liver and growing liver of baby rats illustrates the contradictory results one can obtain (TABLES 1 and 2). This type of bio-assay is further complicated by the fact that the mitotic inhibition is only temporary and may escape detection unless an extensive time study is performed.¹⁰ All the difficulties of this system must be taken into consideration in evaluating the results quoted in TABLE 2, which show an inhibitory effect of injections of parotid homogenates using apparently the same experimental technique as that for liver injections that were ineffective under the same experimental conditions.

Mitotic Stimulation

The presence and organ specificity of mitosis stimulator(s) in different tissues and serum are subject to controversial claims, probably due to the lack of well-

standardized test systems. The basic experiment supporting the idea of mitosis stimulator(s) is the induction of cell divisions in the intact liver of one animal of a pair or triplet of parabiotic rats following partial hepatectomy in the other parabiont.^{11,21} This experiment indicates the transmission of mitosis stimulation by the blood, a conclusion apparently supported by the observation that

TABLE 1
THE INFLUENCE OF LIVER HOMOGENATES ON THE MITOTIC
RATE OF REGENERATING LIVER*

	Amount of homogenate per 100 g. body weight	No. of rats	Mitosis per 1000 cells	
			Average	Range
Partial hepatectomy	0	15	36.2	30.5-38.9
Partial hepatectomy and liver homogenate	0.2 g.	10	47.0	28.8-55.6
	0.4 g.	9	33.8	29.8-37.1
	0.8 g.	10	12.4	8.7-15.3
	1.6 g.	10	26.1	13.6-33.8

* Partial hepatectomies were performed at 10:00 A.M. to 12:00 A.M., the homogenates prepared and injected at 5:00 to 6:00 P.M. on the following day, and the tissues were fixed at 10:00 A.M. of the third day.

TABLE 2
THE INFLUENCE OF HOMOGENATES OF PAROTIS AND LIVER ON THE MITOTIC
RATE OF PAROTIS, KIDNEY, AND LIVER OF BABY RATS*

	Amount of homogenate per 100 gm. body weight	No. of rats	Metaphase per 100 cells		
			Parotis	Kidney	Liver
Control	—	3	22.1	7.8	7.7
Parotis	0.6 gm.	4	4.7	2.3	8.1
Control	—	4	26.7	7.3	3.4
Parotis	0.5 gm.	4	7.3	3.5	3.5
Control	—	4	5.4	4.1	1.7
Parotis	0.6 gm.	5	4.8	2.7	2.2
Control	—	8	17.2	7.1	6.3
Liver	0.6 gm.	7	13.5	5.9	5.8

* The homogenates were injected between 11 and 12 A.M., colchicine was administered 24 hours later and the animals sacrificed 8 hours after the colchicine application.

injections of serum obtained from partially hepatectomized rats stimulated cell division in the intact liver of adult animals.²² This claim was not confirmed by Glinos⁹ under conditions that corresponded to the ones originally reported.²² However, a mitosis stimulation is caused by the serum of partially hepatectomized rats if regenerating liver, following the removal of two lobes, is used instead of the intact liver of an adult rat (TABLE 3), a result¹⁰ that was confirmed and further elaborated by Adibi *et al.*¹²

The question concerning the organ specificity of the mitosis stimulator is of

great interest in this connection. Experiments using tissue cultures of different organs indicated a tissue-specific stimulating effect of serum from partially nephrectomized rats.¹⁴ On the other hand, mitosis was induced in a number of different tissues such as cornea and kidney following partial hepatectomy.²³ The simplest, although strictly hypothetical, interpretation of this result is to assume a general mitotic stimulus induced by partial hepatectomy, the extent of which will be influenced by more organ specific inhibitors. The equilibrium between stimulator and inhibitor,²⁴ which was disturbed by partial hepatectomy, will be more easily restored by intact tissues than by tissues whose total amount was reduced by approximately 70 per cent, as in the liver. This would explain the more extended proliferation of the liver and the single mitotic wave in other tissues, both apparently caused by the same factor, namely, a partial hepatectomy.

TABLE 3
THE INFLUENCE OF SERUM FROM PARTIALLY HEPATECTOMIZED RATS ON THE
MITOTIC RATE OF NORMAL AND REGENERATING LIVER*

	Amount of serum per 100 gm. body weight	No. of rats	Mitosis per 1000 cells	
			Average	Range
Normal adult	0	16	1.7	1.0-2.7
Serum from partially hepatectomized rats	1.5 ml.	10	1.1	0.8-2.4
Partial hepatectomy	0	10	28.3	23.1-33.7
Partial hepatectomy and serum from partially hepatectomized rats	1.5 ml.	10	64.2	49.1-73.4

* The timing of the experiment was the same as described above (TABLE 1).

Systemic Effects of Tumors

Transplantable tumors exert a pronounced influence on other tissues. The induction of cell divisions and of DNA synthesis in the liver is one of the systemic effects of a tumor,^{18,25-27} indicating the transmission of a mitotic trigger by the blood. The effect of serum of tumor bearers on the mitotic rate of regenerating liver was studied in order to elucidate this point.

The sera used in this experiment were obtained from rats bearing Walker 256 carcinoma growing subcutaneously in both axillary regions of the animals. Two groups of tumor-bearing rats were selected as serum donors, one (A) having tumors of diameters 0.5 to 1.0 cm. and the second (B) group with tumors 2.5 to 3.5 cm. in diameter. The blood was drawn from the vena cava, the serum prepared and immediately injected intraperitoneally into partially hepatectomized rats. The results, summarized in TABLE 4, indicate a mitosis-stimulating effect of the serum of Walker tumor bearers, in excess of that obtained by injections of serum of partially hepatectomized rats (TABLE 3).

A quantitative evaluation of individual mitotic stages was undertaken and the results are shown in TABLE 5 expressed as percentage of the total number of mitoses counted. The proportion of telophase to metaphase was in the same magnitude in the regenerating livers of rats injected with serum of Walker 256

carcinoma bearers as in those of the controls. This similarity indicates that the serum administration did not significantly change the timing of individual mitotic phases. The increased mitotic rate, therefore, cannot be caused by an accumulation of cells at the metaphase stage, as occurs for example after colchicine application.

TABLE 4
THE INFLUENCE OF SERA OF WALKER 256 CARCINOMA-BEARING RATS ON THE
MITOTIC RATE OF REGENERATING LIVER*

	Amount of serum per 100 gm. body weight	No. of rats	No. of cells measured per rat	Mitosis per 1000 cells	
				Individual cases	Average
Partial hepatectomy	0	10	18,000	35.3, 34.7, 32.8, 32.6, 32.2, 29.0, 28.4, 28.4, 28.0, 27.0	30.8
Partial hepatectomy plus serum from Walker 256 bearers					
Group A	1.5 ml.	8	25,000	156.0, 128.2, 124.2, 108.9, 89.4, 88.4, 75.3, 63.3	104.2
Group B	1.5 ml.	10	25,000	116.0, 87.5, 86.6, 84.1, 80.2, 79.9, 79.2, 78.6, 66.2, 60.0	81.8

* The timing of the experiment was the same as described above (TABLE 1).

TABLE 5
THE DISTRIBUTION OF INDIVIDUAL MITOTIC STAGES EXPRESSED AS PERCENTAGE
OF THE TOTAL NUMBER OF MITOTIC CELLS COUNTED

	Prophase	Metaphase	Anaphase	Telophase
Partial hepatectomy	22.9	41.9	4.6	30.6
Partial hepatectomy and serum from Walker 256 bearers	27.9	37.2	2.6	31.7

Behavior of Neoplastic Cells

One of the main characteristics of a neoplastic cell is an apparently unlimited proliferation. This raises the question regarding the mechanism that enables the neoplastic cells to escape completely or partially the mitotic regulatory factors of an organism. It is obvious that an answer to this problem requires a more profound knowledge of mitosis regulation under normal conditions. The ideas proposed at the present time therefore must be considered as hardly more than suggestions of different possibilities. Nevertheless it appeared of interest to examine whether the ideas of growth regulation as discussed by Glinos,⁹ and above, might have some bearing on the tumor problem.

The development of a tumor line resistant to 6-mercaptopurine (6-MP) offers a model experiment (Paterson and Stich, unpublished). The original

Ehrlich ascites tumor, which was strongly inhibited by the application of 6-MP, consisted of cells characterized by one metacentric chromosome and by chromosome numbers scattered between 70 and 76. Prolonged application of 6-MP led to the formation of 6-MP-resistant cell lines, which were able to divide in the presence of 6-MP. The resistant cell population consisted predominantly of cells with two metacentric chromosomes and chromosome numbers scattered between 67 and 70, with a peak at 68 chromosomes. This example may illustrate that cells with an altered genotype can escape a mitosis inhibitor.

How closely does this model experiment resemble conditions during carcinogenesis? If a similar pattern exists, it should be expected that neoplastic cells have an altered genom and acquire complete or partial insensitivity to mitosis stimulators and inhibitors. The first expectation can be tested readily by chromosome analysis and DNA measurements of tumor cells. The data so far available show an aneuploid chromosome number and DNA content in a

TABLE 6

THE INDUCTION OF MITOSIS BY PARTIAL HEPATECTOMY IN THE HEPATIC TISSUE OF NORMAL RATS AND RATS FED ON A DAB DIET

	Mitosis per 100 cells					
	Normal rat		DAB-feeding 3 weeks		DAB-feeding 4 months	
	Average	Range	Average	Range	Average	Range
Control	0.018	0.00-0.031	0.021	0.011-0.029	0.14	0.03-0.45
Partial hepatectomy	2.2	1.2-2.6	1.6	1.0-1.9	0.26	0.11-0.60

large number of primary tumors of man²⁸⁻³¹ and animals.³²⁻³³ The similarity to the model experiment therefore is given in this point.

The design of experiments that might prove the second supposition, namely, the unresponsiveness of neoplastic cells to mitotic stimulators and inhibitors, is more difficult. The response of a precancerous liver to the mitosis stimulation of a partial hepatectomy might be of interest in this connection. The proliferation induced by the removal of 2 liver lobes is in the same order in the liver of normal rats and of animals fed for 3 weeks on 4-dimethylaminoazobenzene (DAB). The mitotic rate induced is about 100 times larger than that in normal livers of adult rats. The same type of partial hepatectomy provokes only a slight increase of the mitotic rate in precancerous hepatic tissue (TABLE 6). It appears likely that the hepatic cells have acquired an unresponsiveness to the mitotic stimulation of a partial hepatectomy.

A similar phenomenon is observable during leukemogenesis in different mouse strains. Normal mice of a low-leukemic strain respond with a marked lymphocytosis to injections of isologous or homologous thymus extracts, whereas mice in preleukemic stages develop an unresponsiveness to the lymphocytosis-stimulating factors of the thymus.³⁴

Neoplastic cells apparently can also escape from a mitotic inhibition. The mitotic rate of Novikoff hepatomas is not reduced by injections of liver ho-

mogenates that partially inhibit cell divisions of normal regenerating liver, and mitosis in Walker 256 carcinomas is unaffected by one or two subsequent applications of serum obtained from normal adult rats (TABLE 7). These results, however, might be considered merely as indications, since it is difficult to find a control system to which the results might be compared.

TABLE 7
THE INFLUENCE OF ONE AND TWO SERUM INJECTIONS ON THE MITOTIC RATE
OF WALKER 256 CARCINOMA*

	Metaphases per 1000 cells	
	Individual cases	Average
Control	13.0, 13.3, 14.3, 15.0, 15.0, 15.6, 15.6	14.5
One serum injection 1.5 ml./100 gm.	12.6, 13.6, 15.0, 14.0, 14.6, 13.3, 15.6	14.1
Control	14.7, 16.3, 16.5, 16.8, 17.1, 17.3	16.4
Two sera injections each 1.5 ml./100 gm. 6 hours apart	15.6, 15.1, 16.0, 16.0, 19.0, 16.0	16.3

* Small tissue pieces were taken from the carcinoma growing on the right flank prior to the serum injection (control) and compared with samples taken from the carcinoma on the left flank 12 or 24 hours after the first serum injection.

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STIMULATION OF DNA SYNTHESIS AND MITOSIS BY INJURY*

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The lens epithelium offers many advantages for studies on the mechanism by which injury leads to DNA synthesis and cell division. The lens is completely avascular and is enclosed by a membrane, the lens capsule. Therefore it is isolated rather well from other possible secondary influences. In addition, the lens epithelium is a single layer of cells on the anterior surface, just under the lens capsule. This entire layer of cells can be prepared as a whole-mount (Howard, 1952), and the incorporation of tritium-labelled thymidine by any cell in this layer can be detected by means of autoradiography (Harding *et al.*, 1960). In the normal lens, incorporation of thymidine is confined, for the most part, to certain cells near the periphery of the epithelium. This corresponds to the germinative zone in which mitotic figures appear (von Sallmann, 1952). Fourteen to 16 hours after a mechanical injury to the anterior or central region, cells in the vicinity of the wound begin to incorporate thymidine. At later times, cells at greater and greater distances from the wound begin thymidine incorporation.

Starting at approximately 28 to 32 hours, the cells showing incorporation assume the form of one or more bands that surround the wound. This is evident in the photograph of a rabbit lens epithelium 48 hours after injury, as shown in FIGURE 1. A segment of a band of cells with radioactive nuclei plus cells in adjacent areas are shown under higher magnification in FIGURE 2. A zone of mitotic figures can be found adjacent to the zone of incorporating cells on the side toward the wound. In addition, the cells on this side (toward the wound) appear to be more closely packed. The cells outside the bands of radioactive nuclei (upper right in FIGURE 1 and 2) have the appearance of normal cells as seen in preparations from uninjured lenses.

It would seem that a mechanism that incites cells to division moves out slowly from the site of injury. This is evident from the progressive increase in area of cells undergoing synthesis as a function of time after injury, as well as the relative positions of the bands of synthetic and mitotic cells.

The mechanism by which these cells, at progressively greater distances from the wound, are stimulated to undergo division is not clear. The rate at which the effect spreads possibly may reflect the rate of diffusion of a stimulatory substance(s) from the site of injury, or possibly the way in which a naturally occurring inhibitor to division is diluted or inactivated. It might depend, of course, on an entirely different mechanism such as cell-to-cell interaction.

The lens epithelium, which exists in an enclosed avascular system, may possibly have certain unique advantages for studies on the relation between injury and mitosis. If we are dealing here with soluble stimulatory or inhibitory substances, the probability of their dilution or removal would seem less likely than in other cell systems exposed to extracellular fluids in more active motion.

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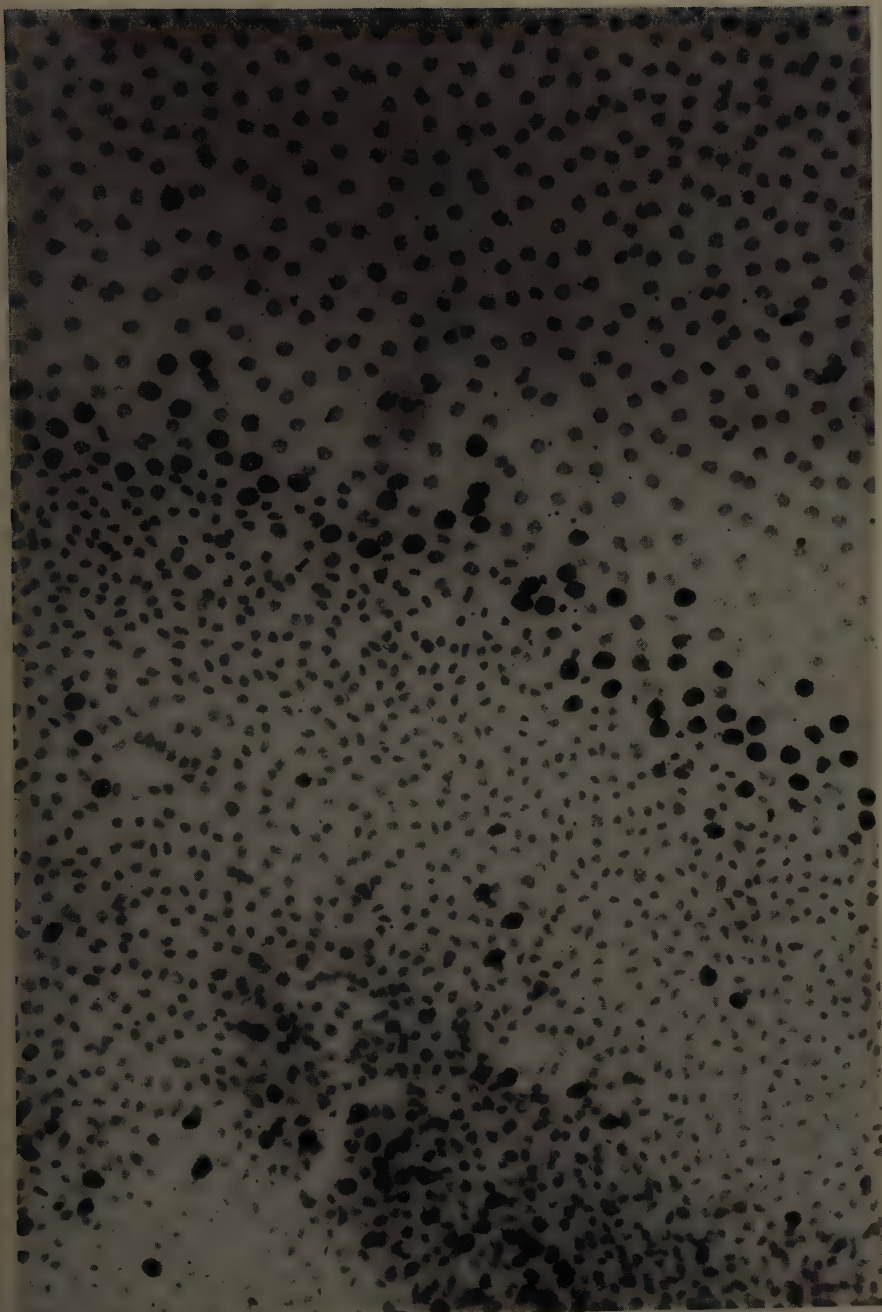


FIGURE 1. Autoradiogram of 48-hour wound in rabbit lens epithelium. Approximately $3\text{ }\mu\text{c.}$ of tritium-labeled thymidine (spec. act., $1.9\text{ curies}/\mu\text{mole}$) in Eagle's basal medium was injected into the anterior chamber two hours before the eyes were fixed. Note the presence of 2 bands of cells with radioactive nuclei (the presence of numerous silver grains appears as a blackened area over each radioactive nucleus). The wound lies out of field toward lower left. Harris Hematoxylin. Final magnification approximately $\times 144$.

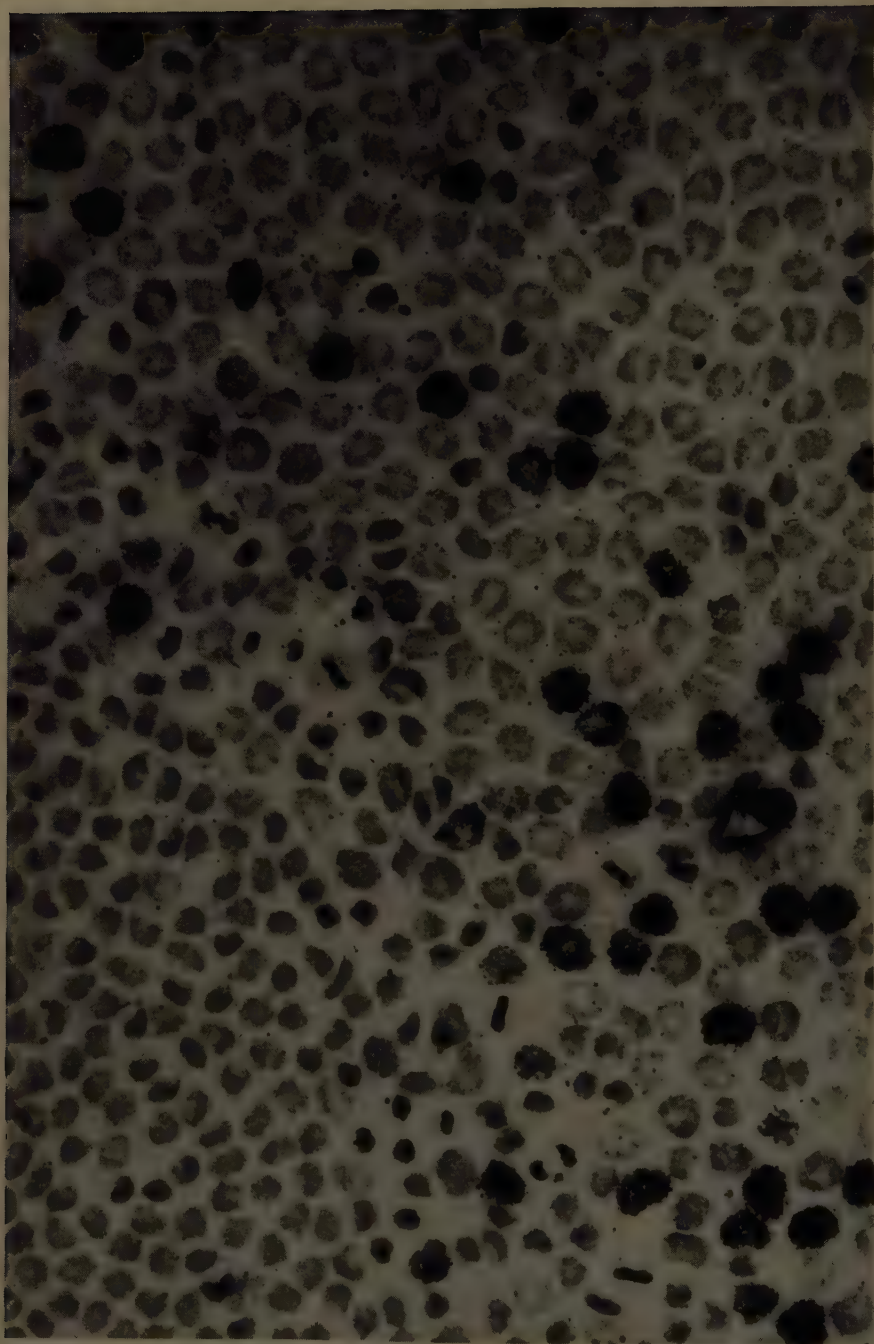


FIGURE 2. Autoradiogram of a 48-hour wound. A zone of mitotic figures is found adjacent to the band of radioactive cells on the side toward the wound (the wound is out of field towards lower left). The mitotic figures themselves are not radioactive. Below and to the left of the radioactive band the cells appear more closely packed. Cells above the radioactive band have the appearance of normal cells from uninjured lenses. Harris Hematoxylin. Final magnification approximately $\times 280$.

From this point of view, it would be of interest to compare the injury reaction in the lens epithelium with the reaction as seen in other cell systems.

References

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